Regulation and evolution of the penicillin biosynthesis gene cluster of *Aspergillus nidulans*

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium

(Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

von Petra Spröte

geboren am 07. April 1980 in Querfurt

2008

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Datum der öffentlichen Verteidigung: 08. Dezember 2008

'I will take the Ring,' he said,

'though I do not know the way.'

J.R.R. Tolkien – The Lord of the Rings

SUMMARY

Penicillin is one of the most important antibiotics and consequently, its biosynthesis is probably the best understood pathway in fungal secondary metabolism. Several studies have indicated that the penicillin biosynthesis gene cluster of the filamentous fungus *Aspergillus nidulans*, comprising the genes *acvA*, *ipnA* and *aatA*, is regulated by a complex network. With AnCF (*A. nidulans* CCAAT-binding factor) and AnBH1 (*A. nidulans* basic region helix-loop-helix protein 1) main *trans*-acting factors were identified. However, there is little knowledge about the environmental signals and their transmission modulating the expression of the structural genes. Moreover, evolution of the gene cluster that contains genes of apparently both bacterial (*acvA* and *ipnA*) and eukaryotic (*aatA*) origin has not been elucidated.

In this work, the light-dependent regulator velvet A (VeA) and a central protein kinase C (PkcA) were found to be part of the penicillin biosynthesis regulating network. Expression of an inducible *alcA*p-*veA* gene fusion indicated that under *alcA*p-inducing conditions both the penicillin titre and *acvA* gene expression were drastically reduced. The analysis of strains with different genetic backgrounds and with a different promoter system (*niiA*p) confirmed VeA as repressor of penicillin biosynthesis in *A. nidulans*, most likely *via* repression of *acvA* gene expression. A putative repressor binding site within the *acvA* promoter was shown not to be involved in VeA-mediated regulation. In conclusion, the obtained findings were assessed in the light of VeA being part of the recently identified *velvet* complex connecting light, development and secondary metabolism.

AnBH1, a repressor of *aatA* gene expression, was shown by *in silico* analysis to contain a putative protein kinase C (PKC) phosphorylation site. Inhibition of PKC activity by application of calphostin C and knocking down of *pkcA* expression by a *pkcA* antisense construct led to cytoplasmic localisation of the usually in the nucleus located AnBH1-eGFP fusion protein, suggesting that PkcA acted upstream of this transcription factor. This finding linked regulation of penicillin biosynthesis to that of cell wall integrity since PkcA appeared to be involved in both signal transduction pathways. However, both nuclear localisation of AnBH1-eGFP and *in vitro* binding of AnBH1 to the *aatA* promoter was independent of the phosphorylated putative PKC site, indicating a rather indirect influence of PkcA.

Analysis of the *aatA* encoded acyl-CoA : isopenicillin N acyltransferase (IAT) by different eGFP fusion proteins and *A. nidulans* mutant strains impaired in peroxisomal biogenesis or function revealed that the peroxisomal localisation of the IAT was dependent on both its atypical peroxisomal targeting signal 1 (PTS1) and the respective PTS1 receptor protein PexE. In contrast to *Penicillium chrysogenum*, in *A. nidulans* strains exhibiting a

mislocalised IAT, penicillin production was reduced but not eliminated. The same was true for strains without functional peroxisomes or with impaired PTS1 import, indicating a beneficial but not essential role of peroxisomes for penicillin production in this fungus.

In contrast to *acvA* and *ipnA* that apparently are of bacterial origin and were most likely obtained by interkingdom horizontal gene transfer, there is no bacterial *aatA* homologue. Thus, the evolutionary origin of *aatA* showing all features of a eukaryotic gene remained obscure. This work showed that disruption of *aatA* still enabled penicillin production in *A. nidulans*. Genome mining led to the discovery of the *aatB* gene which had a similar structure and expression pattern as *aatA*. Phylogenetic analysis revealed putative *aatB* homologues widely distributed also within non-producing fungi, whereas *aatA* homologues were exclusively found as part of the penicillin biosynthesis gene cluster. Disruption of *aatB* in *A. nidulans* resulted in a reduced penicillin titre. In contrast to *aatA*, *aatB* did not encode a PTS1 sequence, resulting in cytoplasmic localisation of the encoded AatB protein. Overexpression of a mutated $aatB^{PTS1}$ gene in an *aatA*-disruption strain, which led to peroxisomal localisation of AatB, increased the penicillin titre more than overexpression of the wild-type *aatB*. Electrophoretic mobility shift assays, surface plasmon resonance analyses and Northern blot analyses indicated that the promoters of both *aatA* and *aatB* were bound and regulated by the same transcription factors AnCF and AnBH1. Taken all these findings together, it was suggested that *aatB* was a paralogue of *aatA*. This thesis therefore extended the model of evolution of the penicillin biosynthesis gene cluster by recruitment of a biosynthesis gene and its *cis*-regulatory sites upon gene duplication.

KURZZUSAMMENFASSUNG

Penicillin gehört zu den wichtigsten Antibiotika und dementsprechend ist die Biosynthese dieses pilzlichen Sekundärmetaboliten besonders gut untersucht. Verschiedene Studien haben gezeigt, dass das Penicillinbiosynthese-Gencluster des filamentösen Pilzes *Aspergillus nidulans,* bestehend aus den Genen *acvA, ipnA* und *aatA,* von einem komplexen regulatorischen Netzwerk kontrolliert wird. Dabei spielen globale Transkriptionsfaktoren wie AnCF (*A. nidulans* CCAAT-bindender Faktor) und AnBH1 (*A. nidulans* basische Region Helix-Loop-Helix-Protein 1) eine wichtige Rolle. Allerdings wurden die Umweltsignale, welche die Expression der Strukturgene mittels bislang ebenfalls unbekannter Signaltransduktionskaskaden beeinflussen, bisher kaum analysiert. Zudem existieren nur wenige Untersuchungen zur Evolution des Genclusters, welches sowohl Gene vermutlich bakteriellen (*acvA* und *ipnA*) als auch eukaryontischen (*aatA*) Ursprungs enthält.

In dieser Arbeit wurden der lichtabhängige Regulator Velvet A (VeA) und eine zentrale Proteinkinase C (PkcA) als Teil des regulatorischen Netzwerks identifiziert. Die Expression einer regulierbaren *alcA*p-*veA*-Genfusion zeigte, dass unter *alcA*p-induzierenden Bedingungen sowohl der Penicillintiter als auch die *acvA*-Genexpression stark reduziert war. Die Analyse von Stämmen mit unterschiedlichem genetischen Hintergrund bzw. mit einem anderen regulierbaren Promotor (*niiA*p) bestätigte den reprimierenden Effekt von VeA auf die Penicillinbiosynthese von *A. nidulans,* welcher vermutlich durch Repression der *acvA*-Genexpression verursacht wurde. Die gewonnenen Erkenntnisse wurden auch vor dem Hintergrund diskutiert, dass VeA kürzlich als Teil des *velvet*-Komplexes identifiziert wurde, welcher lichtabhängig sowohl Entwicklung als auch Sekundärmetabolismus koordiniert.

In silico-Analysen zeigten, dass AnBH1, ein Repressor der *aatA*-Genexpression, eine vermeintliche Phosphorylierungsstelle für eine Proteinkinase C (PKC) enthielt. Sowohl die Inhibition der PKC-Aktivität durch Zugabe von Calphostin C als auch die Expression eines *pkcA*-antisense-Konstrukts beeinflusste die nukleäre Lokalisation einer AnBH1-eGFP-Fusion. Dies ließ auf eine Position von PkcA *upstream* von AnBH1 in der Signalkette schließen. Somit ergab sich eine Verbindung der Regulation der Penicillinbiosynthese mit jener der Zellwandintegrität, da PkcA vermutlich Teil beider Signaltransduktionskaskaden ist. Sowohl die nukleäre Lokalisation von AnBH1-eGFP als auch die *in vitro*-Bindung von AnBH1 an den *aatA*-Promotor waren jedoch unabhängig von einer Phosphorylierung der putativen PKC-Erkennungsstelle, was auf einen indirekten Einfluss der PkcA schließen ließ.

Die Analyse der *aatA*-kodierten Acyl-CoA : Isopenicillin N-Acyltransferase (IAT) mittels verschiedener eGFP-Proteinfusionen und *A. nidulans*-Stämmen mit eingeschränkter

Peroxisomenbildung bzw. -funktion zeigte, dass die peroxisomale Lokalisation der IAT sowohl von deren atypischer peroxisomaler Signalsequenz 1 (PTS1) als auch vom entsprechenden PTS1-Rezeptor PexE abhängig war. Anders als *Penicillium chrysogenum* produzierte ein *A. nidulans*-Stamm mit einer fehllokalisierten IAT immer noch Penicillin. Dies wurde ebenfalls für Stämme ohne funktionelle Peroxisomen bzw. mit eingeschränktem PTS1-Import beobachtet und ließ auf eine vorteilhafte, aber nicht essentielle Rolle der Peroxisomen für die Penicillinbiosynthese in *A. nidulans* schließen.

Im Gegensatz zu *acvA* und *ipnA*, welche wahrscheinlich durch horizontalen Gentransfer aus Prokaryonten auf die Pilze übertragen wurden, gab es kein bakterielles *aatA*-Homolog. Der evolutionäre Ursprung dieses eindeutig eukaryontischen Gens war somit ungeklärt. Diese Arbeit zeigte, dass die Disruption des *aatA*-Gens in *A. nidulans* die Produktion geringer Mengen an Penicillin immer noch ermöglichte. Die anschließende Analyse des Genoms führte zur Entdeckung des strukturell ähnlichen *aatB*-Gens. Phylogenetische Untersuchungen ergaben, dass putative Homologe des *aatB*-Gens auch in nicht-produzierenden Pilzen vorkamen, wohingegen *aatA*-Homologe stets Teil des Penicillinbiosynthese-Genclusters waren. Eine *aatB*-Disruption reduzierte den Penicillintiter in *A. nidulans*. Da *aatB* keine PTS1 kodierte, war AatB im Zytoplasma lokalisiert. Die Überexpression einer $aatB^{PTS1}$-Variante und somit die peroxisomale Lokalisation von AatB in einem *aatA*-Disruptionsstamm erhöhte den Penicillintiter stärker als die Überexpression des unveränderten *aatB*-Gens. Mit Hilfe verschiedener biochemischer und molekularbiologischer Methoden konnte gezeigt werden, dass die Promotoren beider Gene von den gleichen Transkriptionsfaktoren AnCF und AnBH1 gebunden und auch reguliert wurden. Alle Daten wiesen auf einen gemeinsamen evolutionären Ursprung der demnach paralogen Gene *aatA* und *aatB* hin und erweiterten das bestehende Modell der Evolution des Penicillinbiosynthese-Genclusters um die Rekrutierung eines Gens und seiner regulierenden Faktoren nach erfolgter Genduplikation.

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ABBREVIATIONS

Nucleotides and standards amino acids were abbreviated according to the IUPAC (International Union of Pure and Applied Chemistry) and the three-letter code, respectively.

$^{-}$	auxotrophy
$^{+}$	prototrophy
A.	*Aspergillus*
AAA	aminoadipic acid / aminoadipyl
ACV	δ-(L-α-AAA)-L-cysteinyl-D-valine
ACVS	ACV synthetase
AMM	*Aspergillus* minimal media
Amp	ampicillin
AnBH1	*A. nidulans* bHLH protein 1
AnBH1f	AnBH1 (3rd annotation 2006)
AnCF	*A. nidulans* CCAAT binding factor
6-APA	6-aminopenicillanic acid
BA	benzoic acid
β-GAL	β-galactosidase
β-GLU	β-glucuronidase
bHLH	basic region helix-loop-helix
bp	base pair
°C	degree Celsius
C-terminal	carboxy-terminal
cDNA	DNA complementary to mRNA
CoA	coenzyme A
cp	cyclopentanone
CWI	cell wall integrity
Δ	deletion
2D-GE	two dimensional gel electrophoresis
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E.	*Escherichia*
e.g.	for example
eGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility shift assay
et al.	and others
FM	fermentation media
g	gram
Glc	glucose
h	hour
HGT	horizontal gene transfer
HRP	horseradish peroxidase
IAT	acyl CoA : IPN acyltransferase
i.e.	that is
IPN	isopenicillin N
IPNS	IPN synthase
kbp	kilobase pairs
K_D	dissociation constant
kDa	kilodalton
l	litre
LB	Luria broth
Lc	lactose
LC	liquid chromatography
M	molar
MalE	*E. coli* maltose-binding protein
MAP	mitogen-activated protein
min	minute
mRNA	messenger RNA
MS	mass spectrometry
N-terminal	amino-terminal
ORF	open reading frame
p	promoter
P.	*Penicillium*
PA	phenylacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCL	phenylacetyl-CoA ligase
PCR	polymerase chain reaction
pH	negative logarithm of proton concentration
pI	isoelectric point
PKC	protein kinase C
PkcA	*A. nidulans* PKC A
PPase	protein phosphatase
PTS	peroxisomal targeting signal
^{R}	resistance
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RT	reverse transcription
RT	room temperature
RU	response unit
SD	standard deviation
SPR	surface plasmon resonance
U	unit of enzyme activity
VeA	velvet A
VeA1	N-terminally truncated VeA
v/v	volume per volume
wt	wild type
w/v	weight per volume

INTRODUCTION

1. *Emericella (Aspergillus) nidulans*

The aspergilli form a ubiquitous group of filamentous fungi spanning over 200 million years of evolution (Galagan et al., 2005). The genus name *Aspergillus* derives from the similarity in appearance between the microscopic anatomy of the spore-bearing (asexual) conidiophore (Fig. 1A) and an aspergillum, the instrument used for sprinkling holy water in the Roman Catholic Church (Baker & Bennett, 2007). There is probably no other fungal genus that contains both species so beneficial and species so harmful to humans: among the over 185 aspergilli are several that have a serious impact on human health and society (e.g., human pathogens; Steinbach, 2007), as well as advantageous species used to produce foodstuffs and industrial enzymes, prompted by their small physical sizes, haploid genomes, simple nutritional requirements and rapid life cycles (Timberlake & Marshall, 1989). In nature, they are also important as agents of deterioration and decay (summarised in Baker & Bennett, 2007).

Within the genus *Aspergillus*, *A. nidulans* has a central role as a model organism. In contrast to most aspergilli, it possesses a well-characterised sexual cycle with sexual spores (ascospores) and thus a well-developed genetic system. And indeed: its species name *nidulans* – literally meaning "to nest" – refers to the way the ascospore-containing structures (cleistothecia, Fig. 1B) are embedded into mycelia during the sexual cycle. The according to taxonomic nomenclature correct systematic name *Emericella nidulans*, that was given to the fungus after discovery of its sexual life cycle (Samson, 1994), has hardly been established in the scientific community. Therefore, this study will use the traditional name *Aspergillus nidulans*.

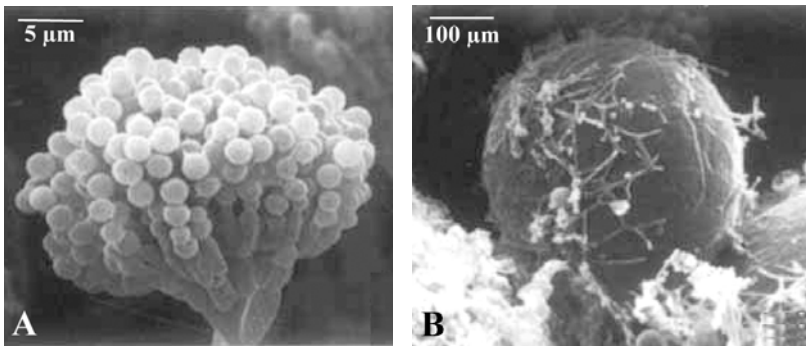

Fig. 1. Scanning electron microscopic images of different developmental stages of *A. nidulans* (modified from Krüger et al., 1997). (**A**) Conidiophore carrying asexual conidia. (**B**) Cleistothecium containing asci with sexual ascospores.

Half a century of *A. nidulans* research – starting with Pontecorvo's work in the 1950s (Pontecorvo et al., 1953) – has advanced the study of eukaryotic (multi-) cellular physiology, contributing to our understanding of metabolic regulation, pH control, DNA repair, controlled RNA degradation, morphogenesis, chromatin structure, cell cycle control, cytoskeletal function, mitochondrial DNA structure and other aspects of eukaryotic genetics and cell physiology (Jones, 2007).

A. nidulans is very well suited for such studies: it is easily grown and manipulated in the laboratory (e.g., Ballance et al., 1983). Its life cycle is mainly haploid producing uninucleate spores and, together with the genetic system, this makes the organism particularly useful both for classical genetic studies and for the application of the new technologies of molecular genetics (Todd et al., 2007). Since 2003, the genome of the species is fully sequenced, up-to-date annotated (Galagan et al., 2005) and open to the public (www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). Therefore, *A. nidulans* has been, and continues to be, one of the most important model organisms for fundamental biological processes – with findings often valid also for higher eukaryotes. In the present study, these features were used to obtain novel insights into regulation and evolution of the penicillin biosynthesis gene cluster.

2. Penicillin

Antibiotics are low-molecular weight substances that inhibit microbial growth already at low concentrations (Lancini & Parenti, 1982). Their discovery is probably the most important one in history of therapeutic medicine, since their application in treating infectious diseases may conceivably have saved more lives than any other medical innovation (Heatley, 1990). Penicillin was one of the first antibiotics to be discovered (Fleming, 1929; Clutterbuck et al., 1932) and it is still one of the most important in terms of both therapeutic use and annual volume of production (Barber et al., 2004). Because of this success resulting largely from the discovery of semi-synthetic derivatives with improved efficacy (Nayler, 1991), a lot of effort has gone into improving the efficiency of penicillin production, resulting in an increase in titre from the 1.2 mg/l of Fleming's original *Penicillium notatum* isolate to 50 g/l and above in modern strains, that are derived from a higher-producing isolate of *Penicillium chrysogenum* (Abraham, 1990; Queener & Swartz, 1979). Nowadays, other antibiotics with different chemical structures and biological targets are both discovered and developed (Skatrud, 1991). However, despite the growing incidence of penicillin-resistant isolates, the β-

lactam antibiotics, which include also cephalosporins, are due to their high specificity and low toxicity still by far the most frequently used antibiotics.

It is yet not clear why fungal strains do produce penicillin, although there is speculation about conferring a selective advantage in the natural habitat or being means of communication. As penicillin is not essential for growth and reproduction under standard laboratory conditions, it is considered as secondary metabolite (Vining, 1990). Production of penicillin is rather energy consuming (Maplestone et al., 1992), and therefore, it must not be a by-product without any benefit for the producer. The biosynthesis of these very complex structures is tightly regulated and catalysed by unique enzymes encoded by genes that are organised in clusters (Brakhage, 1998).

The penicillin pathway is possibly the best (although still incompletely) understood fungal secondary-metabolism pathway (Peñalva et al., 1998). Further investigation – in particular of the regulatory network – is necessary with respect to both medical and economic interests: this could result in the biosynthesis of so far unknown compounds with new therapeutic characteristics (e.g., hybrid antibiotics; Chater, 1990; Weber et al., 1991). Additionally, it may lead to an increase of product yield and might help in the design of optimisation strategies for other economically relevant pathways (Peñalva et al., 1998). For basic research, the penicillin biosynthesis pathway serves as a classical model system for both the analysis of general mechanisms of gene regulation in lower eukaryotes and to get a better understanding of secondary metabolism and its connection to the primary metabolism (Brakhage, 1997). Furthermore, it provides a model for the evolution of secondary metabolite gene clusters (Brakhage et al., 2005). The use of the modest penicillin producer *A. nidulans* as an experimental organism has greatly facilitated these studies, because (in contrast to *P. chrysogenum*) it is amenable to both formal and reverse-genetic techniques – and therefore, *A. nidulans* was and still is preferred.

Although many of the steps and regulatory circuits of penicillin biosynthesis have been characterised, others remain to be identified, and targets for improvement are no longer obvious. To this end, studies on the regulation of gene expression and the cell biology of penicillin biosynthesis in the genetic model organism *A. nidulans* provide the best option for gene identification and isolation (Peñalva et al., 1998). Transfer of this knowledge to *P. chrysogenum* is relatively easy, although not always entirely possible. Furthermore, a deeper analysis of the structural genes, their evolutionary origin and their encoded proteins also contributes to complete our knowledge about this unique and important pathway.

2.1. Penicillin biosynthesis pathway

Penicillins are only produced by filamentous fungi, notably *A. nidulans* and *P. chrysogenum*, but the biosynthesis pathway has the first two steps – and thus the corresponding enzymes and their genes – in common with the cephalosporin biosynthesis pathway that is also found in some bacteria, including Gram-negative and Gram-positive microorganisms. Both penicillins and cephalosporins are synthesised from the same three amino acids, L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine (Fig. 2; reviewed in Brakhage, 1998; Schmitt et al., 2004). In fungi, the non-proteinogenic amino acid L-α-AAA is an intermediate of the fungal-specific aminoadipate pathway leading to the formation of L-lysine (Strassman & Weinhouse, 1953), or can be obtained by catabolic degradation of L-lysine by an ω-aminotransferase (Valmaseda et al., 2005). In the first reaction, the amino acid precursors are condensed to the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) catalysed by a single enzyme, ACV synthetase (ACVS; van Liempt et al., 1989; Theilgaard et al., 1997). This multi-modular enzyme belongs to the group of non-ribosomal peptide synthetases (NRPS; Finking & Marahiel, 2004) and was the first fungal NRPS identified (Smith et al., 1990b).

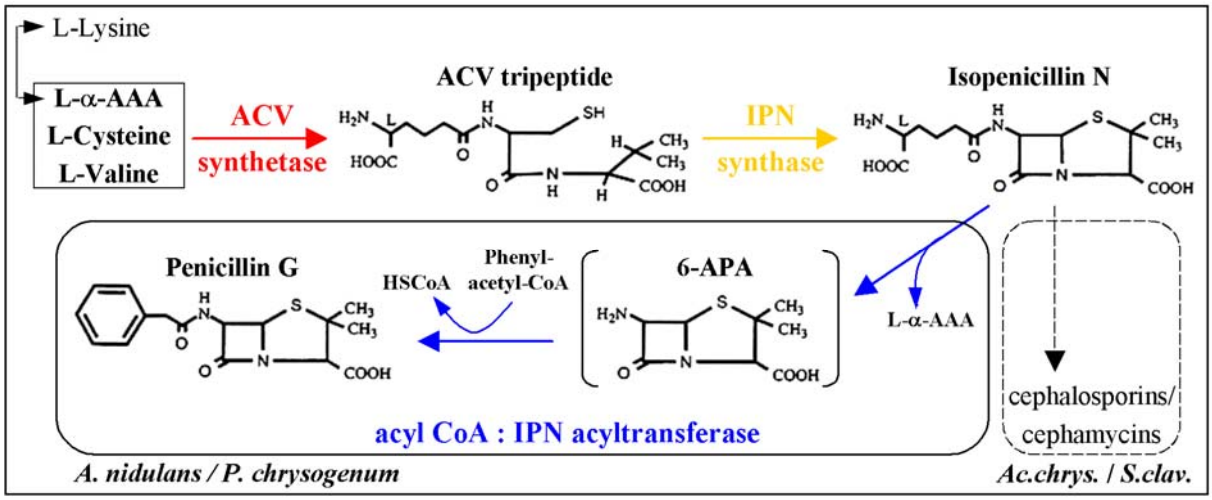

Fig. 2. Penicillin biosynthesis pathway and involved enzymes (see text for details)**.** The branching point to cephalosporin / cephamycin biosynthesis (e.g., by *Acremonium chrysogenum* / *Streptomyces clavuligerus*) is indicated by the dashed arrow and box. Steps involving acyl CoA : IPN acyltransferase (IAT; blue arrows) are penicillin specific and only performed by the filamentous fungi *A. nidulans* and *P. chrysogenum* (framed). Abbreviations: L-α-AAA, L-α-aminoadipic acid; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; IPN, isopenicillin N; 6-APA, 6-aminopenicillanic acid.

In the second step, oxidative ring closure of the linear tripeptide leads to formation of a bicyclic ring, i.e., the four-membered β-lactam ring that the group is named after is fused to the five-membered thiazolidine ring which is characteristic of all penicillins. This reaction is

catalysed by isopenicillin N synthase (IPNS; Ramón et al., 1987). Isopenicillin N (IPN), the first bioactive intermediate possessing weak antibiotic activity, is the branching point of penicillin and cephalosporin biosynthesis (Fig. 2; Brakhage, 1998).

In the third and final reaction of penicillin biosynthesis, the hydrophilic L-α-AAA side chain of IPN is exchanged for a hydrophobic acyl group catalysed by acyl CoA : IPN acyltransferase (IAT; Fernández et al., 2003). Hydrophilic penicillins represent biosynthetic intermediates (e.g., IPN), whereas hydrophobic penicillins are final biosynthetic products which are released and accumulated in the fermentation broth, and moreover, have a higher activity and a broader antibacterial spectrum (Luengo, 1995). Due to IAT activity, the formation of hydrophobic penicillins has been reported in fungi only (indicated in Fig. 2), and there is no obvious homologue in bacteria (Skatrud, 1991). Since IAT possesses a broad substrate specificity, by supplying the cultivation media with phenylacetic or phenoxyacetic acid, the synthesis can be directed towards penicillin G and V, respectively (Luengo, 1995; Alvarez et al., 1993), whereas in nature mainly penicillin DF, F and K occur (Brakhage, 1998).

2.2. IAT – the third enzyme of the pathway

For the conversion of IPN to penicillin G a two-step enzymatic process has been proposed (Queener & Neuss, 1982). In the first step, IPN is deacylated to 6-aminopenicillanic acid (6-APA), and in the second step, 6-APA is acylated to penicillin G through the addition of a phenylacetyl group from its CoA-derivative (Fig. 2). Both steps are performed by acyl CoA : IPN acyltransferase (IAT) that, in *P. chrysogenum*, was shown to have isopenicillin N amidohydrolase, 6-APA acyltransferase, and penicillin amidase activities (Alvarez et al., 1993) with the latter one being the reverse of the biosynthetic 6-APA acyltransferase activity. Due to the broad substrate specificity of IAT, different hydrophobic penicillins are formed dependent on the precursor whose activated form is generally believed to be the CoA-thioester (Fig. 2). In *P. chrysogenum*, two phenylacetyl-CoA ligase-encoding genes were suggested to be involved in the activation of the side chain precursors (Lamas-Maceiras et al., 2006; Wang et al., 2007).

IAT and, thus, production of hydrophobic penicillins are restricted to filamentous fungi, although there is a striking functional similarity to bacterial acylases that catalyse the deacylation of penicillins and cephalosporins (Schumacher et al., 1986). However, there is only very low sequence similarity (approximately 11% identical amino acids) between fungal IATs and the *E. coli* penicillin acylase (Brakhage, 1998). Therefore, no obvious homologues

in bacteria exist. Luengo et al. (1995) speculated that IAT is the only enzyme able to catalyse the last step of penicillin biosynthesis and that its substrate specificity is broad enough to produce more penicillins than those occurring naturally in industrial broths.

Most studies on the IAT protein have been carried out in *P. chrysogenum*. The active form of the IAT enzyme results from processing of the 40 kDa monomeric precursor (375 amino acids) to a heterodimer containing subunits of 11 (corresponding to the N-terminal region) and 29 kDa (C-terminal region) (Alvarez et al., 1987; Tobin et al., 1990). The processing event that is most likely autocatalytic (Tobin et al., 1993) occurs between Gly^{102} and Cys^{103} within the DGC motif of the preprotein (Aplin et al., 1993). Both the *A. nidulans* and the *P. chrysogenum* IAT share 76% sequence identity. Although the DGC motif is conserved, it was suggested that both proteins differ in their processing ability, i.e., the *A. nidulans* IAT maintains the 40 kDa monomer but is still able to catalyse the reaction (Fernández et al., 2003). The authors proposed that this difference may affect the overall penicillin-biosynthesis ability of the two fungi.

Due to its C-terminal peroxisomal targeting signal 1 (PTS1), the IAT of *P. chrysogenum* is located within single membrane organelles, namely peroxisomes (Müller et al., 1991). A mislocalisation of the protein due to a deletion of the PTS1-encoding DNA sequence was shown to fully abolish penicillin production (Müller et al., 1992) demonstrating the necessity of the peroxisomal localisation of IAT in this fungus (Evers et al., 2004). Despite the otherwise high sequence similarity, the *A. nidulans* IAT possesses a different C-terminal tripeptide (-ANI instead of -ARL in *P. chrysogenum*) that is not considered as a typical PTS1 (Swinkels et al., 1992; Brocard & Hartig, 2006). However, cellular localisation of the protein and the role of both this C-terminal sequence and peroxisomes for penicillin biosynthesis in *A. nidulans* have yet not been elucidated in detail. First studies of *A. nidulans* strains with only few peroxisomes indicated less importance of these organelles for penicillin production (De Lucas et al., 1997).

2.3. Clustering of the β-lactam biosynthesis genes

The three different enzymes of penicillin biosynthesis are encoded by the *acvA* (*pcbAB*), *ipnA* (*pcbC*) and *aatA* (*penDE*) gene, respectively, with the former names *pcb* (penicillin and cephalosporin biosynthesis) and *pen* (penicillin specific) for *P. chrysogenum*, and the enzyme-derived names for *A. nidulans*. With the beginning of the 1990s these genes had been cloned and sequenced (Carr et al., 1986; Barredo et al., 1989; Ramón et al., 1987; Weigel et al., 1988; Smith et al., 1990a; MacCabe et al., 1990; Montenegro et al., 1990). Despite the

different nomenclature, the three genes are very similar in *A. nidulans* and *P. chrysogenum*, and the overall organisation into a gene cluster is identical (Fig. 3, upper part; Martin et al., 1997; Montenegro et al., 1992). The *acvA* and *ipnA* homologues are transcribed from the same, bidirectional promoter, whereas *aatA* (*penDE*) possesses its own regulatory sequences (Brakhage & Turner, 1995).

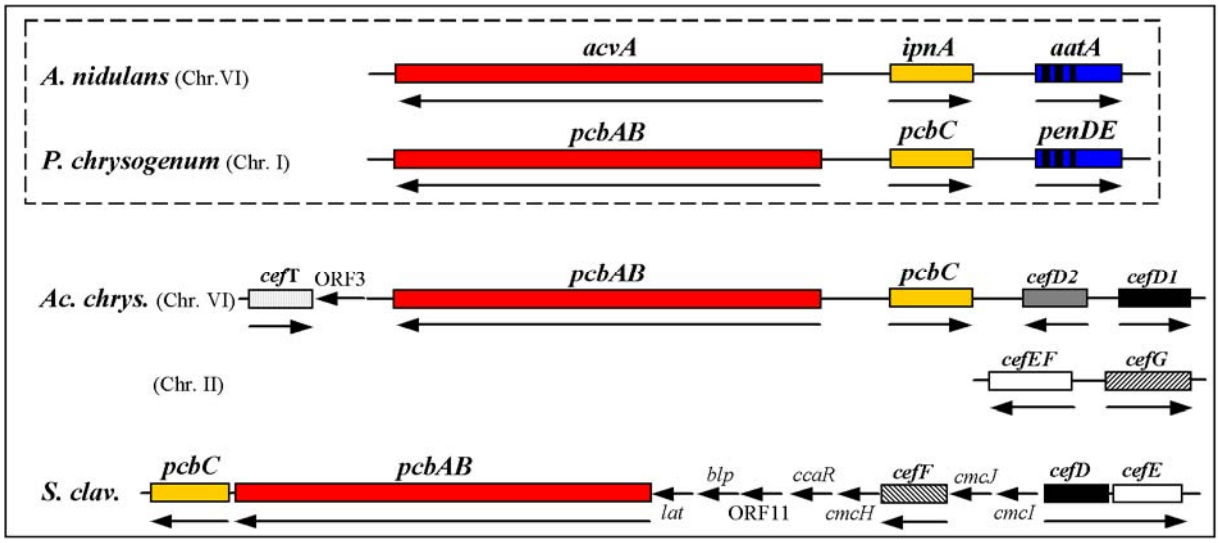

Fig. 3. β-lactam biosynthesis gene clusters in the filamentous fungi *Aspergillus nidulans, Penicillium chrysogenum* and *Acremonium chrysogenum* and in the Gram-positive bacterium *Streptomyces clavuligerus* (reviewed in Brakhage et al., 2004). Data for *A. chrysogenum* were modified according to Schmitt et al., 2004. Chromosome numbers (in fungi) are indicated. Bacterial genes with fungal homologues are boxed; same colours (consistent with Fig. 2) or shading patterns mark homologous genes. Non-coloured genes are involved in cephalosporin and / or cephamycin biosynthesis (for abbreviations see Brakhage et al., 2004). Transcriptional orientation is indicated by arrows. Introns in the *aatA* (*penDE*) gene are marked with black boxes, the other genes do not contain introns. The *aatA* (*penDE*) gene is only present in penicillin producers (upper part, framed with dashed lines), whereas both the *acvA* (*pcbAB*) and the *ipnA* (*pcbC*) gene are found in all β-lactam producing organisms.

The lower part of Fig. 3 shows the biosynthesis gene clusters of other β-lactam producing microorganisms, namely *Acremonium chrysogenum*, a fungal cephalosporin producer (Schmitt et al., 2004), and *Streptomyces clavuligerus*, a Gram-positive cephamycin producer (Liras, 1999). Thus, linkage of the biosynthesis genes is a well-known phenomenon in antibiotic-producing organisms, although in fungi there is no obvious need for clustering. It thus seems more likely that linkage reflects a common ancestral origin (see below). However, there is no evidence that *aatA* has a close relative in modern prokaryotes, even though it is part of the cluster. Therefore, linkage might also confer an ecological advantage to the eukaryotic fungi in their natural habitat, although the reason for this is not yet understood (Brakhage et al., 2005).

3. Evolution of β-lactam biosynthesis genes in fungi

As exemplarily depicted in Fig. 3, β-lactam biosynthesis genes are found in both *some* bacterial and *some* fungal species. Despite the challenging publication of Smith (Smith et al., 1992), interkingdom horizontal transfer is the prevalent hypothesis concerning evolution of these genes (Carr et al., 1986; Weigel et al., 1988; Landan et al., 1990; Peñalva et al., 1990; Aharonowitz et al., 1992). The following arguments in favour of horizontal gene transfer (HGT) have been put forward: (i) *ipnA* genes of fungi and bacteria show high sequence identities; (ii) both bacterial and fungal β-lactam genes are organised in clusters - and the genes for the late stage of cephalosporin biosynthesis (non-coloured in Fig. 3) could presumably have been lost in an ancestor of the penicillin producers; (iii) the unusually high GC content in the third position of codons encoding the fungal *ipnA* genes may indicate an evolutionary origin from streptomycetes; (iv) fungal *acvA* and *ipnA* genes do not contain introns (Fig. 3) indicating a bacterial origin; (v) most of the (higher) eukaryotes and fungi must have lost the genes if they had been distributed *via* vertical descend, which is rather unlikely. Concerning the direction of the HGT, a transfer from bacteria to fungi is more conceivable, since an opposite event would have been lethal for bacteria without the presence of resistance mechanisms (reviewed in Brakhage et al., 2005).

Within this HGT hypothesis, the *aatA* gene has an exceptional position, since there is no obvious homolog in bacteria (Skatrud, 1991), and moreover, it possesses the most characteristic features of a eukaryotic gene: introns and an own promoter (Fig. 3). Therefore, interkingdom HGT for this gene can almost be excluded. Alternatively, it is proposed that, during evolution, *aatA* was recruited to the biosynthesis genes *acvA* and *ipnA* that were left after the loss of the late cephalosporin biosynthesis genes. Isopenicillin N already possesses weak antibiotic activity. However, the recruitment and involvement of IAT to the pathway led to formation of penicillins with much higher antibiotic activity, which could explain the selection pressure to add the third gene *aatA* to *acvA* and *ipnA* (Brakhage et al., 2005). This pressure was not on eukaryotic organisms that still had the complete pathway leading to a fully active antibiotic substance (e.g., to cephalosporin in *A. chrysogenum*; Fig. 3).

Tobin et al. (1990) concluded, that the high degree of identity observed for the *aatA* homologues in *A. nidulans* and *P. chrysogenum* and, moreover, the identical position of all three introns in both genes (Fig. 3) support a common ancestral gene origin. They listed the lack of IAT activity in the prokaryotic β-lactam producers as a further hint that this gene may have evolved from a eukaryotic progenitor. However, until now the evolutionary origin of the *aatA* gene remains obscure.

4. Regulation of penicillin biosynthesis in *A. nidulans*

First insights into molecular mechanisms that regulate penicillin biosynthesis were obtained by simply analysing penicillin production under different conditions. Glucose repression was known from industrial fermentation of *P. chrysogenum* (Soltero & Johnson, 1953), and a similar effect could be observed in *A. nidulans* (Brakhage et al., 1992; Espeso & Peñalva, 1992). However, the molecular basis of that C-source regulation is still not completely understood (Brakhage et al., 2004); a role of CreA, the central regulator of carbon repression in primary metabolism of *A. nidulans* (Bailey & Arst, 1975), seems to be unlikely (Brakhage & Turner, 1995; Espeso et al., 1995). The observation that growth at alkaline pH has a positive effect on penicillin biosynthesis in *A. nidulans* (Shah et al., 1991) led to the identification of the central transcription factor PacC that activates expression of *ipnA* (Tilburn et al., 1995) and *acvA* (Then Bergh & Brakhage, 1998) under alkaline conditions.

However, prediction of other regulatory circuits that are involved in the regulation of penicillin biosynthesis is almost impossible since the physiological meaning of this secondary metabolite for *A. nidulans* is not fully understood. Therefore, in recent years, the alternative strategy of identifying the regulatory proteins first, followed by elucidation of the putatively connected regulatory circuits, was applied (Brakhage et al., 2004). By analysing the promoter regions of the penicillin biosynthesis genes for putative transcription factor binding sites, further parts of the complex regulatory network could be discovered (see below).

All factors identified so far seem to represent wide-domain regulators involved in the regulation of genes of primary metabolism as well. Furthermore, they are not confined to β-lactam producing fungi (Brakhage et al., 2005). This is of particular interest with respect to the evolutionary origin of the regulatory network – it suggests that the penicillin biosynthesis gene cluster, which does not contain pathway specific regulatory genes (Fig. 3), has recruited transcriptional regulators of the primary metabolism by a so far unknown mechanism to also regulate the *acvA*, *ipnA* and *aatA* genes.

4.1. *cis*-acting DNA sequences and *trans*-acting regulators

A variety of *cis*-acting DNA elements and regulatory factors is involved in the regulation of fungal β-lactam biosynthesis. They represent typical eukaryotic proteins (e.g., basic-region helix-loop-helix proteins, CCAAT binding factors or zinc finger proteins) with no indication of a possible prokaryotic origin (Brakhage et al., 2005). In *A. nidulans*, among others like the above mentioned PacC, these regulators include AnCF and AnBH1. Both factors were identified by biochemical methods that followed promoter analyses.

AnCF (*A. nidulans* CCAAT binding factor) is a heterotrimeric protein complex consisting of the subunits HapB, HapC and HapE (Steidl et al., 1999). It was shown to bind to a CCAAT box (I) within the bidirectional promoter of *acvA* and *ipnA*, and to a CCAAT box (II) present in the promoter of the *aatA* gene (Fig. 3) exerting a positive regulatory effect on both *ipnA* and *aatA* gene expression (Litzka et al., 1998). Although it has been estimated that AnCF regulates more than 200 genes (Brakhage et al., 1999), a deletion of subunit-encoding genes of the complex is not lethal (Papagiannopoulos et al., 1996) indicating an involvement of AnCF in the regulation of only a certain subset of genes including some of the penicillin biosynthesis genes. Recently, it was shown that such a subset (e.g., iron dependent genes) can be defined through interaction of AnCF with additional proteins (Hortschansky et al., 2007).

AnBH1 (*A. nidulans* bHLH protein 1) belongs to the family of basic-region helix-loop-helix (bHLH) transcription factors. It binds *in vitro* as a homodimer to an asymmetric variant of the E-box – the classical binding motif of bHLH proteins (Littlewood & Evan, 1994) – within the *aatA* promoter and acts as a repressor of *aatA* gene expression. Since this E-box overlaps with the AnCF binding site (CCAAT box II), AnBH1 counteracts the positive action of AnCF. AnBH1 is expected to be involved not only in regulation of penicillin biosynthesis, because deletion of the *anbH1* gene appears to be lethal for the fungus (Caruso et al., 2002). In the course of the third annotation of the *A. nidulans* genome in 2006, the new *anbH1f* locus (AN7734.3) led to an N-terminally extended version of AnBH1 termed AnBH1f. However, with respect to cellular localisation and regulation of the *aatA* gene there were no differences observed between AnBH1 and AnBH1f (Wolke, 2007).

4.2. Environmental signals and signal transduction cascades

Although substantial research was carried out on the optimisation of penicillin biosynthesis in fungi (Peñalva et al., 1998), until now only a few environmental signals influencing expression of the biosynthesis genes have been identified and further analysed. As detailed above, examples of such external factors are the pH, the carbon and nitrogen sources (the latter only in *P. chrysogenum;* (Haas & Marzluf, 1995)) as well as availability of certain amino acids and oxygen (Brakhage et al., 2004)).

The same deficiencies apply to the signal transduction cascades that link those external signals to the DNA-binding regulators. Since AnBH1 belongs to the bHLH transcription factor family that were all shown to be phosphorylated (Littlewood & Evan, 1994), this protein could be a target of protein kinases. Identifying such an upstream regulatory kinase could be the first step in elucidating the whole signal transduction cascade leading to AnBH1

and, moreover, might give hints on so far undiscovered environmental signals. For example, protein kinases A and C are central components of fundamental signal transduction chains in fungi; i.e., within cAMP mediated signalling (Ni et al., 2005) and MAP kinase cascades (Schmitz & Heinisch, 2003), respectively.

An important environment signal that regulates physiological and developmental processes in *A. nidulans* is light (Mooney & Yager, 1990). The connection to penicillin biosynthesis was set with the observation that the light-dependent regulator velvet A (VeA) of *A. nidulans* influences the steady state transcript levels of *ipnA* and *acvA*. It was postulated that VeA is an activator of penicillin biosynthesis (Kato et al., 2003). VeA was originally found to mediate a developmental light response (Käfer, 1965; Mooney & Yager, 1990; Yager, 1992). An *A. nidulans* strain with the wild-type *veA* allele produces conidia (Fig. 1A) in the presence of red light while it undergoes sexual reproduction forming cleistothecia (Fig. 1B) under permanent dark conditions. Most laboratory strains are *veA1* strains producing a mutated, i.e., truncated, version of the VeA protein. In these strains the dependency on light is diminished: they form conidia even in the dark, whereas sexual reproduction is delayed and reduced (Champe et al., 1981). Strains carrying a full deletion of the *veA* locus show, accompanied by the secretion of a dark pigment (Krappmann et al., 2005), a completely acleistothecial phenotype (Kim et al., 2002), indicating that *veA* is required for cleistothecia and ascospore formation. However, the molecular mechanism by which *veA* regulates sexual development is still unknown, as VeA does not show similarity with any other protein of known function (Calvo et al., 2004). There is speculation that VeA might be part of a complex with other regulators or, at least, interact with light-sensitive proteins (Mooney & Yager, 1990). In addition to its role in developmental processes, VeA is involved in the regulation of secondary metabolism in other Aspergilli (e.g., Calvo et al., 2004; Duran et al., 2007). Homologues of *veA* are found in other filamentous fungi across genera, but not in plants or animals (Kato et al., 2003).

As mentioned above, Kato et al. (2003) reported that penicillin production of *A. nidulans* was reduced in a Δ*veA* strain compared with the level produced in a wild-type control strain. The authors suggested that VeA was necessary for transcription of the *acvA* gene, but acted as a repressor of *ipnA* gene expression. Because groups working on the penicillin biosynthesis in *A. nidulans* usually used *veA* mutant strains which produced considerable amounts of penicillin and *acvA* transcripts (e.g., MacCabe et al., 1990; MacCabe et al., 1991; Brakhage et al., 1992; Brakhage & Van den Brulle, 1995; Pérez-Esteban et al., 1995; Mingot et al., 1999), a more detailed analyses of the influence of VeA on the penicillin

biosynthesis is necessary to elucidate this discrepancy. Taken together, VeA seems to connect development and secondary metabolism, and therefore, light might be another environmental signal that is involved in the regulation of penicillin biosynthesis.

5. Aims of the work

Penicillin is one of the most important antibiotics and consequently, its biosynthesis is probably the best understood pathway in fungal secondary metabolism. However, this knowledge also sets the basis to be – for the first time – able to fully understand both regulation, particularly *via* so far unknown external signals, and evolution of a gene cluster. It was therefore the objective of this work to obtain novel insights into these aspects of penicillin biosynthesis in *A. nidulans*.

Light is known as an important environmental signal for this fungus. Since there have been contradictory observations, a more biochemical approach using different *veA* overexpression systems and reporter gene fusions of the penicillin biosynthesis genes should help to elucidate the influence of the light-dependent regulator VeA on regulation of penicillin biosynthesis in *A. nidulans*. Upstream regulators can be identified by analysing the amino acid sequence of already known transcription factors. Since AnBH1 possesses a putative protein kinase C (PKC) phosphorylation site (J. Heinisch, personal communication), the role of both this site and a putative PKC for nuclear localisation of AnBH1 should be investigated using the green fluorescent protein (GFP) as a marker (Suelmann et al., 1997). A putative involvement of PKC would provide clues to further, so far unconnected signal transduction cascades and their corresponding external stimuli.

A deeper characterisation of IAT, the enzyme that catalyses the final step of penicillin biosynthesis, was expected to give a hint to the evolutionary origin of its encoding *aatA* gene. This characterisation should also include studies on the subcellular localisation of IAT and the importance of functional peroxisomes for penicillin production of *A. nidulans*. The identification of a putative paralogous gene in *A. nidulans* would raise the interesting question concerning its connection to *aatA*. Consequently, this would require comparative analyses of the paralogues in terms of structure and functional domains, *cis*-acting sequences and regulation, phylogeny as well as importance for penicillin production. Data obtained by applying a variety of biomolecular, biochemical and bioinformatic methods could eventually contribute to the existing model of evolution of the penicillin biosynthesis gene cluster and its regulating network.

MATERIALS AND METHODS

1. Strains, media and microbiological methods

1.1. Table 1. Bacterial strains

Strain	Description	Reference
Escherichia coli		
TOP10F'	F' [*lac*I^{q}Tn10 (Tet^R)], *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ80d/*lacZ*ΔM15, Δ*lacX74*, *recA1*, *deoR*, *araD*139, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*(Str^R), *endA1*, *nupG*	Invitrogen
DH5α	F$^-$, Φ80d/*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *recA1*, *endA1, hspR17*($r_k^-m_k^+$), *supE44*, λ$^-$, *thi-1*, *gyrA96*, *relA1*	Invitrogen
Bacillus calidolactis		
C953	wild type	Brakhage et al., 1992

1.2. Table 2. *Aspergillus nidulans* strains

Strain	Description	Reference
AXB4A2	*veA1*; *pyrG89*, *pabaA1*; *argB2; fwA1*; *bga0*; *argB2*::pAXB4A (*acvA*p-*uidA*, *ipnA*p-*lacZ*); ArgB$^+$	Weidner et al., 1998
AX a-veA	AXB4A2; pAL4-veA; PyrG$^+$	This study
AX n-veA	AXB4A2; pKTB-niiAp-veA; PyrG$^+$	This study
OVAR5	*pabaA1*, *yA2*; *trpC801*; Δ*argB*::*trpC*; *veA1*, pVEL-nA (*niiA*p-*veA*, *argB*); TrpC$^+$, ArgB$^+$	Kim et al., 2002
ΔveA	*pabaA1*, *yA2*; *trpC801*; Δ*argB*::*trpC*; Δ*veA*::*argB*; TrpC$^+$, ArgB$^+$	Kim et al., 2002 (=DVAR)
ΔveA a-veA	ΔveA; pAL4-veA; pabaAnid; PabaA$^+$	This study
ΔCCA-G URA	*veA1*; *pyrG89*, *biA1*; *bga0*; *argB2*, *argB2*::pΔCCA-G [*acvA*p(mut)-*uidA*, *ipnA*p(mut)-*lacZ*]; ArgB$^+$	This study
ΔCCA-G a-veA	ΔCCA-G URA; pAL4-veA; PyrG$^+$	This study
LOGOAnBH1	*pyrG89, biA1; fwA1; bga0; argB2*:: pOLAB1 (*aatA*p-*lacZ*); pRG1; pLOGO-AnBH1 (*anbH1-egfp*); ArgB$^+$, PyrG$^+$	Herrmann et al., 2006
R21	*yA2, pabaA1*	Fantes & Roberts, 1973
AnBH1(S71A)-GFP	R21; p123-anbH1-S71A; Paba$^+$	This study
AnBH1(S71E)-GFP	R21; p123-anbH1-S71E; Paba$^+$	This study
11.4	*yA2, pabaA1*; *anbH1*::*alcA*p-*anbH1*	Caruso et al., 2002
11.4-AnBH1(S71A)	11.4; p123-anbH1-S71A; Paba$^+$	This study
HapB-eGFP	*pyrG89*, *pabaA1*; *fwA1*; *bga0*; *argB2*::pAXB4A (*acvA*p-*uidA*, *ipnA*p-*lacZ*); pHapB-GFP (*hapB-egfp*); ArgB$^+$, PyrG$^+$	Steidl et al., 2004

C2.3	*pyrG89*, *biA1; fwA1*; *bga0; argB2*:: pOLAB1 (*aatA*p-*lacZ*); pLOGO-AnBH1 (*anbH1-egfp*); ArgB$^+$	Herrmann et al., 2006
PkcAinv1.3	*pyrG89*; *fwA1*; *bga0; biA1; argB2*:: pOLAB1 (*aatA*p-*lacZ*); pLOGO-AnBH1 (*anbH1-egfp*); (pAL4-pkcAinv)$_n$; ArgB$^+$, PyrG$^+$	Herrmann et al., 2006
TN02A21 (Δ*nkuA*)	*pyroA4; nkuA::argB; riboB2*	FGSC A1150
AatA-disr	TN02A21; *aatA*::pbar-aatA-disrupt; glufosinate^R	This study
A-disr_aatA	aatA-disr; pAfpyro-aatA_natp (*aatA*p-*aatA*); Pyro$^+$	This study
A-disr_ovA	aatA-disr; pAL4(pyro)-aatA (*alcA*p-*aatA*); Pyro$^+$	This study
A-disr_ovB	aatA-disr; pAL4-aatB(pyro) (*alcA*p-*aatB*); Pyro$^+$	This study
A-disr_ovB^{PTS1}	aatA-disr; pAL4-aatB^{PTS1}(pyro) (*alcA*p-*aatB*^{PTS1}); Pyro$^+$	This study
AatB-disr	TN02A21; *aatB*::pAfribo-aatB-disrupt; Ribo$^+$	This study
B-disr_aatB	aatB-disr; pAfpyro-aatB_natp (*aatB*p-*aatB*); Pyro$^+$	This study
AatA/B-disr	*pyroA4; nkuA::argB*; *aatA*::pbar-aatA-disrupt; *aatB*::pAfribo-aatB-disrupt (progeny from cross AatA-disr × AatB-disr)	This study
AatA$^{\Delta PTS1}$	TN02A21; *aatA*::pbar-aatA_delPTS; glufosinate^R	This study
A$^{\Delta PTS1}$ /B-disr	*pyroA4; nkuA::argB*; *aatA*::pbar-aatA_delPTS; *aatB*::pAfribo-aatB-disrupt (progeny from cross AatA$^{\Delta PTS1}$ × AatB-disr)	This study
AX_GFP-IAT	AXB4A2; pALX-aatA; pabaAnid; Paba$^+$	This study
AX_GFP-AatB	AXB4A2; pALX-aatB; pabaAnid; Paba$^+$	This study
AX_GFP-AatB^{PTS1}	AXB4A2; pALX-aatB^{PTS1}; pabaAnid; Paba$^+$	This study
ΔE-89	*pyrG89, yA2, pabaA1;* Δ*hapE::argB;* ArgB$^+$	Steidl et al., 2004
AnBH1f-GFP	*pabaA1, yA2;* p123-AnBH1f; Paba$^+$	Wolke, 2007
A234	*yA2, pabaA1*	FGSC A234
GFP-IAT	A234; pALX-aatA; pabaAnid; Paba$^+$	This study
GFP-IAT$^{\Delta PTS1}$	A234; pALX-aatA$^{\Delta PTS1}$; pabaAnid; Paba$^+$	This study
pexC::bar	*pyroA4*; *pexC::bar*	Hynes et al., 2008
pexC::bar GFP-IAT	*pexC::bar*; pALX-aatA; SM6363; Pyro$^+$	This study
*pexE*Δ	*yA2, pabaA1*; *pexE::riboB*	Hynes et al., 2008
*pexE*Δ GFP-IAT	*pexE*Δ; pALX-aatA; pabaAnid; Paba$^+$	This study
pexG14	*yA2, pabaA1*; *pexG14* (point mutation)	Hynes et al., 2008
pexG14 GFP-IAT	*pexG14*; pALX-aatA; pabaAnid; Paba$^+$	This study
Aspergillus oryzae	wild type	DSM 1862

1.3. Table 3. List of plasmids

Name	Relevant genotype	Reference
pCR2.1TOPO	Amp^R	Invitrogen
pabaAnid	Amp^R, *pabaA*	Tüncher et al., 2005
pAL4	Amp^R, *pyr-4*, *alcA*p	Waring et al., 1989
pAL4-veA	Amp^R, *pyr-4*, *alcA*p-*veA*	This study
pKTB1	Amp^R, *pyr-4*	Then Bergh, 1997
pKTB-niiAp-veA	Amp^R, *pyr-4*, *niiA*p-*veA*	This study
pBlueScript SK+	Amp^R	Stratagene
pJET1/blunt	Amp^R	Fermentas
p123	Amp^R; *otef*p-*egfp*	Spellig et al., 1996
p123-anbH1-S71A	Amp^R; *otef*p-*anbH1*(S71A)-*egfp*, *pabaA*	This study
p123-anbH1-S71E	Amp^R; *otef*p-*anbH1*(S71E)-*egfp*, *pabaA*	This study
pMAL-c2X	Amp^R; *malE* (encoding maltose binding protein of *E. coli*)	New Engl. Biolabs
pMAL-anbH1	Amp^R; *malE-anbH1*	Caruso et al., 2002
pMAL-anbH1-S71A	Amp^R; *malE-anbH1*(S71A)	This study
pMT1612	Amp^R; *amdS*p-*bar*	Monahan et al., 2006
SM6355 (*bar*)	Amp^R; *amdS*p-*bar*, intact MCS	gift from M.J. Hynes
pbar-aatA-disrupt	Amp^R; *amdS*p-*bar*, *aatA* disruption construct	This study
pbar-aatA$^{\Delta PTS1}$	Amp^R; *amdS*p-*bar*, *aatA*$^{\Delta PTS1}$ construct (*aatA* lacking the PTS1 encoding sequence) for replacing *aatA*	This study
SM6363 (*Af-pyro*)	Amp^R; AFUA_5G08090[1], intact MCS	gift from M.J. Hynes
pAfpyro-aatA_natp	Amp^R; AFUA_5G08090[1], *aatA*p-*aatA*	This study
pAfpyro-aatB_natp	Amp^R; AFUA_5G08090[1], *aatB*p-*aatB*	This study
SM6392 (*Af-ribo*)	Amp^R; AFUA_1G13300[2], intact MCS	gift from M.J. Hynes
pAfribo-aatB-disrupt	Amp^R; AFUA_1G13300[2], *aatB* disruption construct	This study
pAL4(pyro)	Amp^R; AFUA_5G08090[1], *alcA*p	This study
pAL4(pyro)-aatA	Amp^R; AFUA_5G08090[1], *alcA*p-*aatA*	This study
pAL4-aatB(pyro)	Amp^R; *alcA*p-*aatB*, AFUA_5G08090[1]	This study
pAL4-aatB^{PTS1}(pyro)	Amp^R; *alcA*p-*aatB*^{PTS1}, AFUA_5G08090[1]	This study
pALX213	Amp^R; *gpdA*p-*egfp*	Szewczyk et al., 2001
pALX-aatA	Amp^R; *gpdA*p-*egfp*-*aatA*	This study
pALX-aatA$^{\Delta PTS1}$	Amp^R; *gpdA*p-*egfp*-*aatA*$^{\Delta PTS1}$	This study
pALX-aatB	Amp^R; *gpdA*p-*egfp*-*aatB*	This study
pALX-aatB^{PTS1}	Amp^R; *gpdA*p-*egfp*-*aatB*^{PTS1}	This study

[1]AFUA_5G08090: pyridoxine biosynthesis gene from *A. fumigatus* complementing *pyroA4*

[2]AFUA_1G13300: riboflavin biosynthesis gene from *A. nidulans* complementing *riboB2*

1.4. Standard media and supplements

LB-medium (-agar): 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, (15 g/l agar). If required, 50 µg/ml ampicillin was added.

Aspergillus minimal medium (AMM) (-agar): 6 g/l $NaNO_3$, 1.52 g/l KH_2PO_4, 0.52 g/l KCl, pH 6.5, (15 g/l agar). After autoclaving the following was added: glucose to a final concentration of 0.8 % (w/v), 0.05 % (w/v) $MgSO_4$, and 1 µl/ml trace element solution. Trace element solution: 1 g $FeSO_4 \times 7H_20$, 8.8 g $ZnSO_4 \times 7H_2O$, 0.4 g $CuSO_4 \times 5H_2O$, 0.15 g $MnSO_4 \times 4H_2O$, 0.1 g $NaB_4O_7 \times 10H_2O$, 0.05 g $(NH_4)_6Mo_7O_{24} \times 4H_2O$, dd$H_2O$ ad 1,000 ml, sterile-filtrated. This medium was also used for *alcA*p-repressing conditions. For *alcA*p-inducing conditions, glucose as carbon source was replaced by lactose (1.6 % w/v), and 10 mM cyclopentanone was added to the media. For growth under *niiA*p-repressing conditions, $NaNO_3$ as *niiA*p-inducing compound was replaced by 0.2 % (w/v) ammonium tartrate as the nitrogen source (Kim et al., 2002). If required, supplements were added as follows: glufosinate (25 µg/ml; obtained by chloroform extraction from a commercial herbicide; Nayak et al., 2006), pyridoxine-HCl (0.5 µg/ml), riboflavin (2.5 µg/ml), uracil/uridine (2.2 mg/ml and 1 mg/ml, respectively), and *p*-aminobenzoic acid (3 µg/ml). For PKC inhibition, Calphostin C (Biomol, Germany) was applied at a concentration of 5 µM.

1.5. Penicillin bioassay

Fermentation experiments of *A. nidulans* to determine penicillin titres were carried out in fermentation medium (FM; 1 % $CaCO_3$, 2 % corn steep solids, 0.7 % KH_2PO_4, 0.05 % phenoxyacetic acid, 4 % lactose, 0.45 % $MgSO_4$ [all (w/v)]; pH 6.0). Cultures (20 ml in 250 ml shake flasks) were inoculated with same amounts of spores ($3\text{-}5\times10^7$) and incubated at 28°C and 250 rpm. The supernatants were analysed by bioassay (Smith et al., 1989) using *Bacillus calidolactis* C953 as indicator organism. For determination of dry weights, harvested and washed mycelia were dried at 60°C until relative mass constancy. If necessary, p-values showing statistic significance were calculated using student's t-test.

For induction of *alcA*p, 10 mM cyclopentanone were added; for repression, lactose as carbon source was replaced by 4 % (w/v) glucose. Because of the *niiA*p-repressing amounts of ammonia within the corn steep solids, this compound was reduced to 0.2 % (w/v) when *niiA*p was applied as inducible system. These experimental cultures contained 0.2 % (w/v) ammonium tartrate as nitrogen source under *niiA*p-repressing, and 0.3 % (w/v) $NaNO_3$ under *niiA*p-inducing conditions.

1.6. Sexual crosses of *A. nidulans*

Sexual crosses and characterisation of the resulting progeny were performed as described previously (Pontecorvo et al., 1953; Todd et al., 2007).

2. Molecular genetics methods

2.1. Cloning and *in vitro* enzymatic modifications of DNA

Standard cloning procedures, i.e., restriction digests, removal / attaching of 5'-phosphate groups and ligation of compatible DNA ends were performed according to the recommendations of the manufacturers (New England Biolabs, Bioline, Roche Diagnostics; all in Germany). Amplification of thus generated plasmids was performed in *E. coli* after transformation according to the $CaCl_2$ method described by Sambrook et al. (1989).

2.2. Isolation of *E. coli* plasmid DNA

Rapid isolation was performed by alkaline lysis (Birnboim & Doly, 1979). Larger and finer preparations were carried out by means of the "EZNA Plasmid Miniprep Kit", supplied by Peqlab (Germany), according to the manufacturer's instructions.

2.3. Isolation of *A. nidulans* chromosomal DNA and Southern hybridisation

Genomic DNA of *A. nidulans* mycelia grown in AMM was isolated either by the method of Lee and Taylor (Lee & Taylor, 1990) or by using the MasterPure™ Yeast DNA Purification Kit from Biozym Scientific (Germany) according to a modified isolation protocol (Wickes, 2004). For Southern blot analysis, DNA was digested with an appropriate enzyme, separated on an agarose gel (Southern, 1975), and transferred to a Hybond N^+ nylon membrane (GE Healthcare, Germany) by capillary blotting (Sambrook et al., 1989). For detection of the UV-crosslinked DNA fragments on the membrane, either an [α-^{32}P]dATP-labelled DNA probe (Monahan et al., 2006), or the DIG High Prime Labelling and Detection System (GE Healthcare, Germany) with a non-radioactive labelled DNA probe was used according to the manufacturer's instructions.

2.4. Isolation of *A. nidulans* total RNA and Northern hybridisation

Cultures of *A. nidulans* were incubated in AMM or FM under specified incubation conditions. Total RNA was prepared using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. The amount of RNA was determined

spectrophotometrically using a NanoDrop ND-1000 (ThermoFisher Scientific, Germany). For Northern blot analyses, 10 µg of denatured RNA was separated on a formaldehyde-containing [2 % (v/v)] agarose gel, and transferred to a Hybond N^+ nylon membrane (GE Healthcare, Germany) by capillary blotting (both according to Sambrook et al., 1989). The DIG High Prime Labelling and Detection System (GE Healthcare, Germany) using a non-radioactive labelled DNA probe was used for detection of specific mRNAs.

2.5. Isolation of DNA fragments from an agarose gel

Re-isolation of DNA from agarose gels was performed using the Invisorb Spin DNA Extraction Kit (Invitek, Germany) according to the manufacturer's instructions.

2.6. Polymerase chain reaction (PCR) and RT-PCR

Amplification of specific DNA fragments for cloning or for use as hybridisation probes was carried out by PCR. For this purpose, the BIOTAQ Red DNA polymerase (Bioline, Germany) or alternatively, the proofreading Phusion DNA polymerase (New England Biolabs, Germany) was used. Reverse Transcription (RT)-PCR to generate and amplify cDNA from previously isolated RNA was performed using the Bioscript One-Step RT-PCR Kit (Bioline, Germany). The cycling reactions were carried out in a "Personal Cycler" (Biometra, Germany). Elongation time and annealing temperature were chosen to match the oligonucleotide primers and the size of the amplification product.

Table 4. Oligonucleotides used in PCR. Restriction sites for cloning are underlined.

Name	Sequence (5' to 3')
niiAp_KpnI	TCG GTA CCT CTT ATC TCT GG
probe_a-veA_3'	AAG TTG GCA TTG TAG ACG AAC G
probe_a-veA_5'	CTA AGT CCC TTC GTA TTT CTC C
veA_3'_KpnI	ACA GGG TAC CCA GTA GTC AG
veA_3'_PstI	CTG CAG GCA CAC ATC TAT CCA GC
veA_5'_KpnI	GGT ACC GCT ACA CTT GCA GCA CC
anbH1-gfp/Bam	CGC GAG GAT CCG AGC ATG AAC CAA AAA CCG
anbH1-Ala1	CTC TGC TCC CAT CGT TCT GCC ATC TGC CTC
anbH1-Ala2	GAA AAG AGG CAG ATG GCA GAA CGA TGG GAG
anbH1-gfp/Nco	TAT ACC ATG GTC TGA TCA ACG TCG AGC GGC
S71A_BamHI-5'	GGA TCC ATG AAC CAA AAA CCG ACT GTC GG
S71A_SalI-3'	GTC GAC CTT TTA TAC CTG TGT CTG ATC AAC G
aatA-disrupt_5'	CTG GCC GAG ATA CTA TGA GG
aatA-disrupt_3'	GTG GTT CGT ATG CAC TAT CC
aatAp_up	ATT GCA CAG GCC CTT TTC GC
aatA_down	TCT AGA CTC ACA AGG TTA GGA AGG
aatA_up_BglII	AGA TCT ATG CTT CAC GTA ACT TGC CAA GG

aatA_up_BamHI	GGA TCC ATG CTT CAC GTA ACT TGC C
aatA_down_BamHI	GGA TCC TTC GTG AGT CCT AGG TAG G
aatA_exon4_up	TTC TTC ACC GCA ACC AAA G
aatA_exon4_down	TCA AAT GTT GGC TTG GAT CG
aatA_invPCR_delPTS	GAT CCA ATG AAA CAT TTG ACC
aatA_invPCRup	GCG GAC TTG GTA TCC AGA TTG C
aatB-disrupt_5'	TGA CTG GCC TCA GGT ACA GG
aatB-disrupt_3'	GAC GAG GTG AAC TCG AGT CC
aatBp_up	AGG CTG AGG GTG CTA AAA ATG C
aatB_down	AAG GAT ACC TGG TTG GAT GAC G
aatB_up_XmaI	CCC GGG TAT GGC CGT CAA GCA AAT CG
aatB_up_KpnI	GGT ACC GCC GTC AAG CAA ATC GTT TGC
aatB_down_XbaI	TCT AGA GGA TAC CTG GTT GGA TGA CG
aatB-invPCR-PTS	AAC ATT TAG TTG TCG AGG GTA ATC G
aatB-invPCRup	TGC AAG GAT GAC CCT CTC C
aatB_exon4_up	TGG GAT ACC GAA CAA GCC
aatB_exon4_down	CTC AAA TGC AAG GAT GAC CC
AnidaatB_up	CTC CTT CGA TAA GTA TGG
AnidaatB_down	ATT ACC CTC GAC AAC TAC
AoryzaatB_up	TAG ATA CTC CCA CAA TGC
AoryzaatB_down	TCA CGA CAA CAT CTT CAC

Synthesis of oligonucleotides and DNA sequencing was carried out by MWG (Germany).

2.7. *In vitro* site-specific mutagenesis

The method of generation of point mutations by PCR was described by Higuchi (Higuchi et al., 1988). In brief, two sets of oligonucleotide primers are used: complementary primers at the end of the sequence and mismatched primers designed to introduce a point mutation. Two independent PCR reactions employing a proofreading DNA polymerase are performed, each using a primer binding at the end of the sequence and a mutagenic primer with introduced base substitution mutations. A mixture of both PCR products is afterwards denatured by heating to 95°C, and after annealing, this DNA is used as a template for a new PCR employing the outer primers. The resulted PCR fragment is a full-length product containing the required point mutation(s). This method was used to generate the amino acid exchanges at position 71 of AnBH1.

Another method to introduce point mutations or small deletions can be applied if the respective sequence is part of a plasmid. By inverse PCR, either with mismatching primers carrying the intended mutation or with matching primers whose binding leads to exclusion and therefore deletion of parts of the plasmid during the following PCR reaction, the whole plasmid is amplified and afterwards re-ligated. This method was employed to delete the PTS1 of IAT and to introduce a PTS1 into AatB, respectively.

2.8. Transformation of *A. nidulans*

A. nidulans strains were transformed to uracil, *p*-aminobenzoic acid, pyridoxine or riboflavin prototrophy and to glufosinate resistance, respectively, according to the method previously described (Ballance & Turner, 1985).

3. Biochemical methods

3.1. Preparation of crude extracts from *A. nidulans*

Cultures of *A. nidulans* were incubated in AMM or FM under specified incubation conditions. The fungal mycelia were harvested by filtration over miracloth, dried briefly, frozen in liquid nitrogen and pulverised using a mortar and pestle. The resulting mycelial powder was mixed either with protein extraction buffer for subsequent enzymatic assays (see 3.3.) or with 50 mM Tris-Cl pH 8.0 for other applications. In the latter case, the mixture was briefly incubated on ice and afterwards centrifuged for 5 min at 13,000 rpm and 4°C. The supernatant, containing the soluble *A. nidulans* proteins, was retained.

3.2. Determination of protein concentration

Protein concentration was determined according to Bradford (Bradford, 1976) using Roti-Nanoquant (Carl Roth, Germany).

3.3. β-Glucuronidase (β-GLU) and β-galactosidase (β-GAL) activity assays

β-GAL and β-GLU activities were determined in crude extracts (see 3.1.). Incubation of strains and measurements of activities were repeated at a minimum of three times. Specific activities were calculated as previously described (Brakhage et al., 1992).

3.4. SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

SDS-PAGE was performed for the electrophoretic separation of complex protein mixtures as described previously (Laemmli, 1970). The 4-12 % (w/v) bis-Tris SDS polyacrylamide gels (type NuPAGE) were supplied by Invitrogen (The Netherlands). Protein samples were denatured for 5 min at 95°C in NuPAGE loading dye. After cooling briefly on ice, the samples were loaded on the gel and run at 200 V. Prestained PAGERuler (Fermentas, Germany) and BenchMark (Invitrogen) were used as a protein markers.

3.5. Western blot analysis

To analyse sizes of GFP-tagged proteins, proteins from crude extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes *via* semidry blotting. The membranes were blocked with 2.5 % (w/v) bovine serum albumine (BSA) in 1× RotiBlock (Carl Roth, Germany) / phosphate-buffered saline (PBS) with 0.1 % Tween-20 (v/v) for 1 h at room temperature (RT). Monoclonal mouse α-GFP (ab1218 from Abcam, UK) at a dilution of 1:1,000 was applied as primary antibody for 2 h at RT. After five washes with PBS-0.1 % Tween-20 (v/v), horseradish-peroxidase (HRP-) conjugated rabbit α-mouse (P0260 from DakoCytomation, Germany) at a dilution of 1:2,500 was used as secondary antibody for 1 h at RT. After five washes with PBS-0.1 % (v/v) Tween-20, the proteins were detected using enhanced chemiluminescence (Applichem, Germany).

3.6. Source of recombinant proteins

In this work several recombinant proteins were analysed, e.g., in binding studies. Most of them were products of previous and parallel studies and, therefore, were generated and provided by colleagues. The short version of AnBH1 (based on the old annotation of the *A. nidulans* genome) was produced as a MalE (maltose-binding protein of *E. coli*) fusion that could be cleaved by Factor Xa (New England Biolabs, Germany) proteolytic digest (Caruso et al., 2002). According to this, a mutated AnBH1(S71A) was generated in this work. After the third annotation, the N-terminally extended AnBH1f was purified *via* His_6-tag by Daniel H. Scharf. The Hap subunits of AnCF, either MalE- (HapC) or His_6-tagged (HapB, HapE), were derived from the work of Peter Hortschansky (Hortschansky et al., 2007).

3.7. Dephosphorylation of purified proteins

Phosphate groups from serine, threonine and tyrosine residues of recombinant (MalE-) AnBH1 were removed by Lambda Protein Phosphatase (λ-PPase; New England Biolabs, Germany) according to the manufacturer's instructions.

3.8. Small-scale two-dimensional (2D) gel electrophoresis

To test whether λ-PPase treatment changes size and isoelectric properties of AnBH1, the protein was analysed by small-scale (7 cm) 2D gel electrophoresis previously established for *A. fumigatus* proteins (Kniemeyer et al., 2006). 20 µg of the (un-)treated protein mixture was suspended in a lyses buffer solution (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.8 % (w/v) ampholytes, 60 mM DTT, 40 mM Tris), compatible with first dimension isoelectric focusing

(IEF). The protein sample was applied *via* anodic cup loading to 2D gel electrophoresis. The IPG strips were rehydrated overnight in 125 µl rehydration buffer (7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 0.5 % (v/v) IPG buffer, 20 mM DTT). IEF was performed using an IPGphor apparatus (GE Healthcare, Germany) at 20°C and a current of 50 µA/strip, with a linear 14 h gradient reaching 5,000 V, then maintaining 2 h with 5,000 V. Prior to the second dimension run, the strips of 7 cm covering a non-linear pH range of pH 4-7 were reduced for 15 min in equilibration buffer, containing 1 % (w/v) DTT, and then alkylated for 15 min in the same buffer containing 2.5 % (w/v) iodacetamide. The second dimension electrophoresis (SDS-PAGE) was performed on a Minigel-Twin system (Biometra, Germany).

3.9. Electrophoretic mobility shift assay (EMSA)

EMSAs with (mutated) *aatB* promoter fragments as DNA probes and AnCF or AnBH1f as protein were performed by using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. 10 fmol of 5'-biotinylated DNA and 4 pmol of recombinant protein diluted in 10 mM Tris-Cl pH 7.5 were applied. For generation of the DNA duplexes, the respective oligonucleotides (Table 5; Biomers, Germany) were annealed in TES buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 50 mM NaCl) by heating to 95°C and subsequent slow cooling down to room temperature.

Similarly, to analyse the binding capacity of S71A-mutated AnBH1 to the wild-type *aatA* promoter, 5'-fluorescein-labelled fragments (MWG, Germany) were used as DNA probes and detected by HRP-conjugated α-fluorescein antibodies (GE Healthcare, Germany).

3.10. Surface plasmon resonance (SPR) analysis

Real-time analysis of DNA-protein interaction was performed on a Biacore 2000 system at 25°C. Commercially available SA sensor chips were used having a layer of a thin gold film coated with carboxymethyldextran hydrogel matrix to which streptavidin was crosslinked. Data were processed with the BIAevaluation software version 4.1 (all hard- and software purchased from Biacore, Germany). The running buffer used for DNA immobilisation and SPR assay was 10 mM HEPES pH 7.4, 0.15 M NaCl, 1 mM DTT, 0.005 % (v/v) surfactant P20. The buffer was freshly prepared, filtered through a 0.22 µm membrane and degassed prior to use. Refractive index errors due to bulk solvent effects were corrected by subtracting responses from the non-coated flow cell 1. 5'-Biotinylated DNA duplexes (5 nM) containing the (mutated) CCAAT and E-box binding sites from the *aatA* and *aatB* promoter regions (annealed from oligonucleotides in Table 5; Biomers, Germany) were immobilised on two SA

sensor chips on flow cells 2 (wt), 3 ($CCAAT_{mut}$) and 4 ($E\text{-}box_{mut}$) by injection at a flow rate of 10 µl/min until 50 response units (RU) had been bound. AnCF was preformed from the single HapC, HapE and HapB proteins by mixing 0.1 mM solutions of each subunit. Lyophilised AnBH1f was dissolved in water to a final concentration of 0.1 mM. Samples for SPR analysis were generated by 500-fold dilution of these stock solutions in running buffer, followed by serial two-fold dilution. Concentration series from 3.13 to 200 nM for AnCF and from 3.13 to 100 nM for AnBH1f, respectively, were examined for each DNA duplex. Sample injection and dissociation time was set to 2.5 min at a flow rate of 20 µl/min. Regeneration of sensor chips was achieved by treatment with running buffer containing additionally 0.5 M NaCl for AnBH1f and 0.5 M NaCl + 0.01 % (w/v) SDS for AnCF, respectively, for 1 min. Dissociation constants were calculated from the concentration-dependent steady-state binding using the 1:1 steady-state affinity model.

Table 5. Oligonucleotides used for binding studies. (Mutated) boxes are underlined (CCAAT) or **bold (E-box)**. B means 5'-biotin and F 5'-fluorescein labelling, respectively. Generated DNA duplexes are separated by dashed lines.

Name	Sequence (5' to 3')
Shift1	F-TCTTGATGCTCACCAGCCAA**TCACAGG**TTCTCCAAGAAGCACTCGGGA
Shift2	F-GATCCCGAGTGCTTCTTGGAGAA**CCTGTGA**TTGGCTGGTGAGCATCAA
AatA2(104)	TCTTGATGCTCACCAGCCAA**TCACAGG**TTCTCCAAGAAGCACTCGGGATC
AatA2(105)	B-GATCCCGAGTGCTTCTTGGAGAA**CCTGTGA**TTGGCTGGTGAGCATCAAGA
AatAMC2(106)	TCTTGATGCTCACCAGGATC**CCACAGG**TTCTCCAAGAAGCACTCGGGATC
AatAMC2(107)	B-GATCCCGAGTGCTTCTTGGAGAA**CCTGTGG**GATCCTGGTGAGCATCAAGA
AatAMBH2(108)	TCTTGATGCTCACCAGCCAA**GACAAGC**TTCTCCAAGAAGCACTCGGGATC
AatAMBH2(109)	B-GATCCCGAGTGCTTCTTGGAGAA**GCTTGTC**TTGGCTGGTGAGCATCAAGA
AatB641	GGGTCGGCAAGAAAA**CAGGTG**TGGGGGAAACCAATCAAATTCGACATAGA
B-AatB641i	B-TCTATGTCGAATTTGATTGGTTTCCCCCA**CACCTG**TTTTCTTGCCGACCC
AatB641-mutC	GGGTCGGCAAGAAAA**CAGGTG**TGGGGGAAAGATCCCAAATTCGACATAGA
B-AatB641-mutCi	B-TCTATGTCGAATTTGGGATCTTTCCCCCA**CACCTG**TTTTCTTGCCGACCC
AatB641-mutE	GGGTCGGCAAGAAAA**GAGTGT**TGGGGGAAACCAATCAAATTCGACATAGA
B-AatB641-mutEi	B-TCTATGTCGAATTTGATTGGTTTCCCCCA**ACACTC**TTTTCTTGCCGACCC

4. Analytical methods

4.1. Penicillin V extraction from culture supernatants

Supernatants from FM cultures were adjusted to pH 2.0 with 1 N HCl, and penicillin V was extracted by adding *n*-butyl acetate (3 × ⅓ vol.). The organic phase was concentrated

under reduced pressure using a "Laborota 4003" rotary evaporator (Heidolph Instruments, Germany). The pellet was dissolved in 100 µl of Milli-Q water prior to LC-MS analysis.

4.2. Liquid chromatography / mass spectrometry (LC-MS) analysis

For high performance LC (HPLC) separation, an analytical 4.6 x 250 mm (5 µm) Hyperclone™ ODS C18 (Phenomenex, Torrance, CA, USA) column was used. After an isocratic phase of 20 min with 85 % (v/v) buffer A (30 mM ammonium acetate pH 5.0 and 5 % (v/v) acetonitrile) and 15 % (v/v) buffer B [buffer A + acetonitrile 20:80, (v/v)] a linear gradient started, reaching 95 % (v/v) buffer B in 30 min, then maintaining 95 % (v/v) buffer B for 5 min. The injection volume was 20 µL, the flow rate 0.6 ml/min. Penicillin V was identified by retention time obtained for the pure substance (Sigma-Aldrich, Germany) and by MS spectrum. It was monitored using a photodiode array detector and by electro spray ionisation (ESI) mass spectrometry on an LCQ bench-top mass spectrometer equipped with an ion trap mass analyser. For instrument control, data acquisition and data analysis the "Xcalibur" package, version 1.3, was employed. All hard- and software for LC-MS was purchased from ThermoFisher Scientific, Germany.

5. Microscopy

For fluorescence microscopic studies and documentation, a Leica DM4500 B digital fluorescence microscope with GFP filter cube and a Leica DFC480 digital camera (both Leica Microsystems, Germany) were employed. Photographs were processed with Photoshop 7.0 software (Adobe Systems, San José, CA, USA).

6. Databases and bioinformatics

Similarity searches were performed using the *A. nidulans* database of the Broad Institute (www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html) and the Central *Aspergillus* Data Repository (CADRE) database (www.cadre-genomes.org.uk), respectively. Searches for conserved domains of proteins were carried out by the NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer & Bryant, 2004). For alignments of amino acid sequences, the computer program "AlignX" using a modified ClustalW algorithm (Chenna et al., 2003) as module of the "VectorNTI Advance 9.1" software was employed. Phylogenetic trees were generated with the help of ClustalX / NJplot (Jeanmougin et al., 1998) using bootstrap neighbour joining with the number of bootstrap trials = 1,000.

RESULTS

1. Velvet A (VeA)

1.1. Published data and lab experience – an inconsistency

It was previously published that the light-dependent regulator VeA of *A. nidulans* is required for *acvA* gene expression and thus for penicillin production (Kato et al., 2003). As indicated in the introduction, most of the studies dealing with the regulation of penicillin biosynthesis use strains which encode a 36 amino acids N-terminal truncated VeA1 protein (Fig. 4). Since this mutation causes a failure to respond to the light stimulus, strains show asexual development also in the dark, e.g., in the incubator, and therefore, they are easier manageable. These strains do produce penicillin.

The alignment in Fig. 4 shows that the putative bipartite nuclear localisation sequence (NLS) of VeA spanning amino acids Lys^{28} to Arg^{44} is partially missing in VeA1. Recently, the mainly cytoplasmic localisation of VeA1 in *A. nidulans* was confirmed, whereas VeA showed light-dependent accumulation in the nucleus (Stinnett et al., 2007).

Fig. 4. Amino acid sequence alignment of the N-terminal regions of *A. nidulans* VeA and VeA1. VeA1 is identical with VeA but lacks the first 36 amino acids due to a mutation of the start codon. The bipartite NLS of VeA is boxed.

Hence, in the used *veA1* mutant strains, the regulator that is, according to Kato et al. (2003), needed for penicillin biosynthesis is not located within the nucleus and might thus not act properly on transcriptional regulation. Moreover, from the differences in light-response it is conceivable that VeA1 has also lost properties of VeA that might involve cytoplasmic signalling pathways leading to the proposed activation of penicillin production. Nevertheless, those strains lacking the wild-type VeA do produce considerable amounts of penicillin. To elucidate this obvious contradiction, in this work several inducible *veA*-expressing systems were established and analysed in both *veA1* and Δ*veA* strains of *A. nidulans*.

1.2. Generation of a *veA1* strain with inducible *alcA*p-*veA* expression (AX a-veA)

To study the influence of VeA on penicillin biosynthesis gene expression and penicillin production of *A. nidulans* in different genetic backgrounds, plasmid pAL4-veA encoding a

regulatable *alcA*p-*veA* fusion was generated. The practicability of the *A. nidulans* alcohol dehydrogenase gene (*alcA*) promoter for inducible expression of regulatory genes was often demonstrated previously (e.g., Marhoul & Adams, 1995; Caruso et al., 2002; Vienken et al., 2005). The system is repressed by glucose and can be easily induced by cyclopentanone (Waring et al., 1989). For generation of pAL4-veA, a 2.2 kbp DNA fragment including the *veA* gene (except for the start ATG) plus 0.4 kbp of the 3' region was amplified from chromosomal DNA of *A. nidulans* strain OVAR5 (Table 2; Kim et al., 2002) using oligonucleotides veA_5'_KpnI and veA_3'_PstI encoding a *Kpn*I and a *Pst*I site, respectively (Table 4). After sub-cloning, sequencing and restriction with *Kpn*I and *Pst*I, the fragment was cloned into the *Kpn*I/*Pst*I-digested pAL4 vector to yield plasmid pAL4-veA with the *veA* gene plus 3' region under the control of the *alcA* promoter (Fig. 5A). In addition, this plasmid contained the *Neurospora crassa pyr-4* gene which was used as selectable marker to transform the *A. nidulans veA1* mutant strain AXB4A2 (Table 2) to uracil prototrophy.

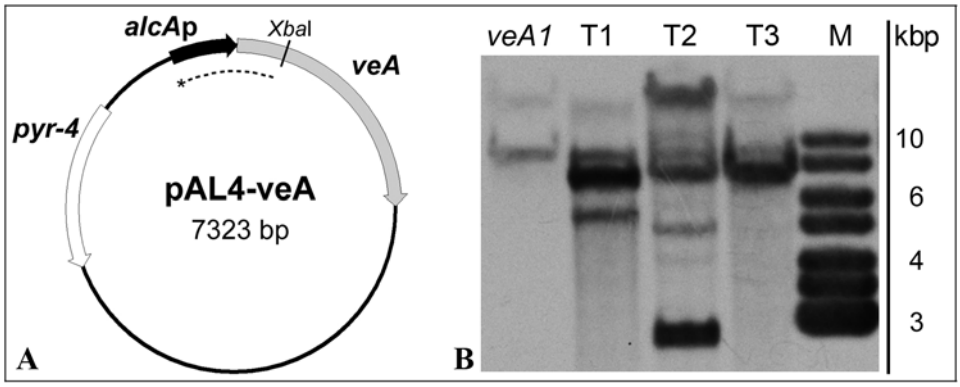

Fig. 5. Generation of an *A. nidulans veA1* strain with inducible *alcA*p-*veA* expression. (**A**) Schematic map of the *alcA*p-*veA*-encoding plasmid pAL4-veA. Abbreviation: *pyr-4*, orotidine-5'-monophosphate decarboxylase gene from *N. crassa* used as selectable marker in *A. nidulans*. Indicated by * is the probe used for Southern blot analysis shown in (B) that was generated by PCR amplification of a 0.8 kbp *alcA*p-*veA* fragment using the oligonucleotides probe_a-veA_5' and probe_a-veA_3', and therefore, also detected the endogenous *alcA*p and the *veA1* gene. (**B**) Southern blot analysis of selected transformants T1-T3 and the untransformed *veA1* strain AXB4A2 using the probe indicated in (A). Genomic DNA was digested with *Xba*I. Transformed strains still showed wild-type bands, but additional signals were indicative of ectopic integrations of the *alcA*p-*veA* cassette.

After transformation, 30 PyrG$^+$ transformants were isolated, and most of them were checked by Southern blot analysis for the presence of the plasmid integrated into the genome (exemplarily shown for the three transformed strains T1-T3 in Fig. 5B). Because the probe employed (in Fig. 5A labelled by *) also hybridised with the endogenous *alcA* promoter and the *veA1* gene, the same bands were detected in both the wild-type strain and the transformants. However, in the transformant strains additional signals were indicative of

ectopic integration of the *alcA*p-*veA* fusion into the genome. One of the transformants (T3 in Fig. 5B) was designated AX a-veA and used for further studies. The intensity of the additional band indicated that in this strain several copies of the gene fusion had integrated into the genome. Because the size of the band matched the size of plasmid pAL4-veA, it is likely that in AX a-veA the plasmid had integrated in tandem repeats at one locus.

VeA mediates a developmental light response (Yager, 1992). Since strains with the wild-type *veA* allele repress asexual development and start to undergo sexual reproduction, they form only few asexual conidia when grown in the dark, whereas *veA1* mutant strains still sporulate under these conditions. To test whether induction of the *alcA* promoter and thus *veA* gene expression in strain AX a-veA could restore this sporulation phenotype that is impaired in the *veA1* strain AXB4A2, both strains were grown under *alcA*p-inducing and -repressing conditions in the dark (Fig. 6). With *alcA*p repressed (glucose), strain AX a-veA sporulated like *veA1* strain AXB4A2. By contrast, when grown under *alcA*p inducing conditions (lactose plus cyclopentanone), AX a-veA in comparison to AXB4A2 showed reduced conidiation, increased formation of aerial hyphae and early steps of sexual development (Fig. 6), which altogether was similar to previous observations of *veA* overexpression (Kim et al., 2002) although formation of cleistothecia started later than expected.

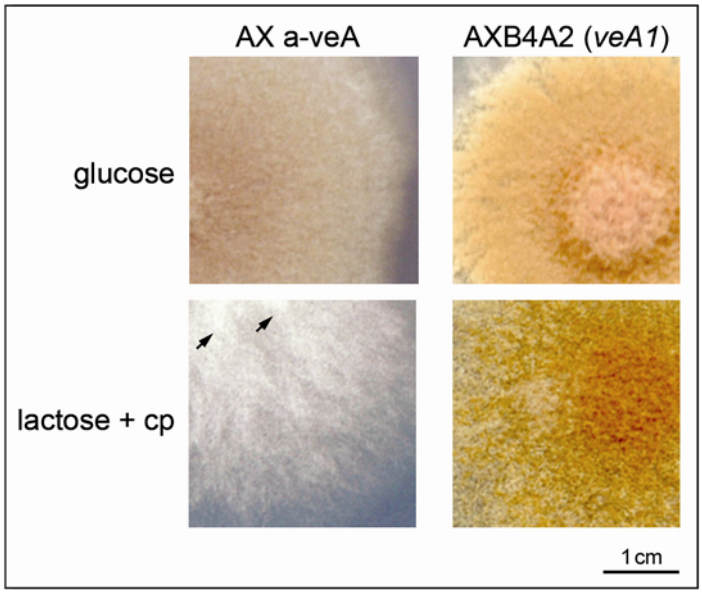

Fig. 6. Phenotypic characterisation of *A. nidulans* strains AX a-veA (left) and AXB4A2 (right). Upper panels: *alcA*p-repressing conditions [0.8 % (w/v) glucose as carbon source]. Lower panels: *alcA*p-inducing conditions [1.6 % (w/v) lactose as carbon source plus 10 mM cyclopentanone (cp)]. Growth and sporulation on AMM agar plates incubated at 37°C for 72 h in the dark. Conidia are brown due to the *fwA1* mutation of the strains (Table 2), and were only formed in the absence of (*alcA*p-driven) *veA* gene expression. Arrows indicate zones of condensed mycelia with beginning cleistothecia formation.

Therefore, in presence of *veA* gene expression, asexual development was repressed whereas sexual development was induced. Taken together, these data showed that expression of *veA* could be induced and that VeA was functional in strain AX a-veA.

1.3. Influence of *veA* overexpression on expression of the penicillin biosynthesis genes and on penicillin production

Since the functionality of the *alcA*p-*veA* system was confirmed, in the next step the influence of *veA* expression on the expression of the penicillin biosynthesis genes was analysed. Therefore, strains AXB4A2 (*veA1*) und AX a-veA (*alcA*p-*veA*) were grown in AMM under *alcA*p-inducing and -repressing conditions for 24 h at 37°C in the dark. The strains also encoded reporter gene fusions of the first two genes of penicillin biosynthesis, *acvA*p-*uidA* and *ipnA*p-*lacZ,* integrated in single copy at the chromosomal *argB* gene locus (Table 2). Protein extracts of mycelia were obtained. β-Glucuronidase (β-GLU) and β-galactosidase (β-GAL) specific activity as marker for *acvA* and *ipnA* gene expression, respectively, were determined (Fig. 7).

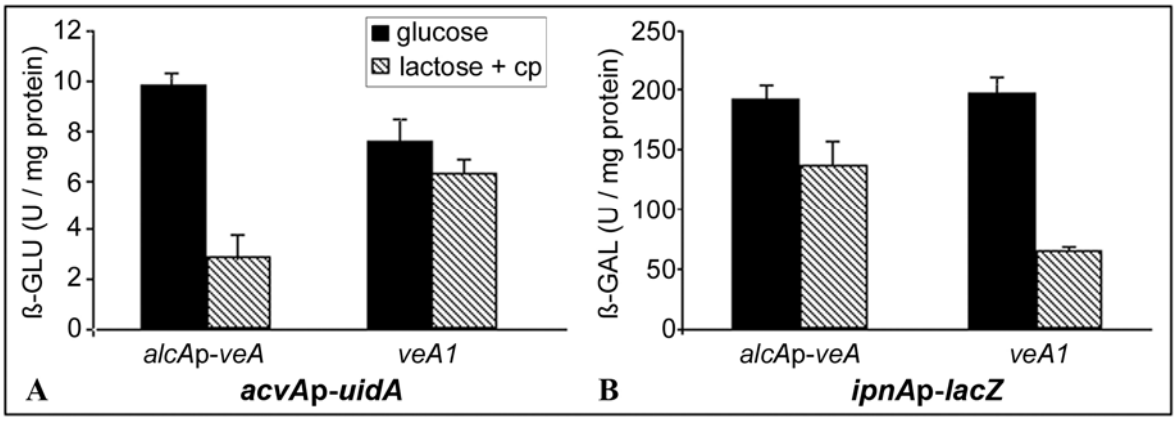

Fig. 7. Expression of *acvA*p-*uidA* (A) and *ipnA*p-*lacZ* (B) gene fusions in strains AX a-veA (*alcA*p-*veA*) and AXB4A2 (*veA1*). Incubation of the strains in AMM under *alcA*p-repressing (■) and *alcA*p-inducing (☒) conditions for 24 h in the dark. Data of each time point represent the mean and SD of three simultaneously harvested flasks. Expression was determined as β-GLU (*acvA*p-*uidA*) and β-GAL (*ipnA*p-*lacZ*) specific activity.

In strain AX a-veA, *acvA*p-*uidA* expression was reduced to about 30 % under *alcA*p-inducing conditions, i.e., in presence of VeA. By contrast, in the *veA1* strain AXB4A2, no significant difference in expression of the *acvA*p-*uidA* gene was observed when the strain was grown under repressing or inducing conditions (Fig. 7A). This suggests a repressing effect of VeA on *acvA* gene expression, whereas the role for *ipnA* is not that obvious. Since *alcA*p-inducing conditions led to a clear decrease of *ipnA*p-*lacZ* expression in both strains (Fig. 7B), the modification of the carbon source in the media had already an effect on *ipnA* expression.

To assess the impact of VeA under fermentation conditions and to also analyse the penicillin titres, the measurements were repeated with strains grown in fermentation media (FM) at 27°C under both *alcA*p-inducing and -repressing conditions over a period of 72 h in the dark (Fig. 8A-C). As a control, the same analysis was carried out in the presence of white light to asses possible light-mediated post-transcriptional regulation of VeA (Fig. 8D-F). It was conceivable that VeA was controlled at this level since its expression was shown to be almost constitutively, i.e., not tightly regulated (Kim et al., 2002). Moreover, in cultures exposed to light the *veA* gene product was shown to be located mainly in the cytoplasm (Stinnett et al., 2007) and might therefore not act on transcriptional regulation. However, due to the muddy consistency of FM, the dense growth of the strains and the nature of submerse cultures, the influence of light was expected to be only weak.

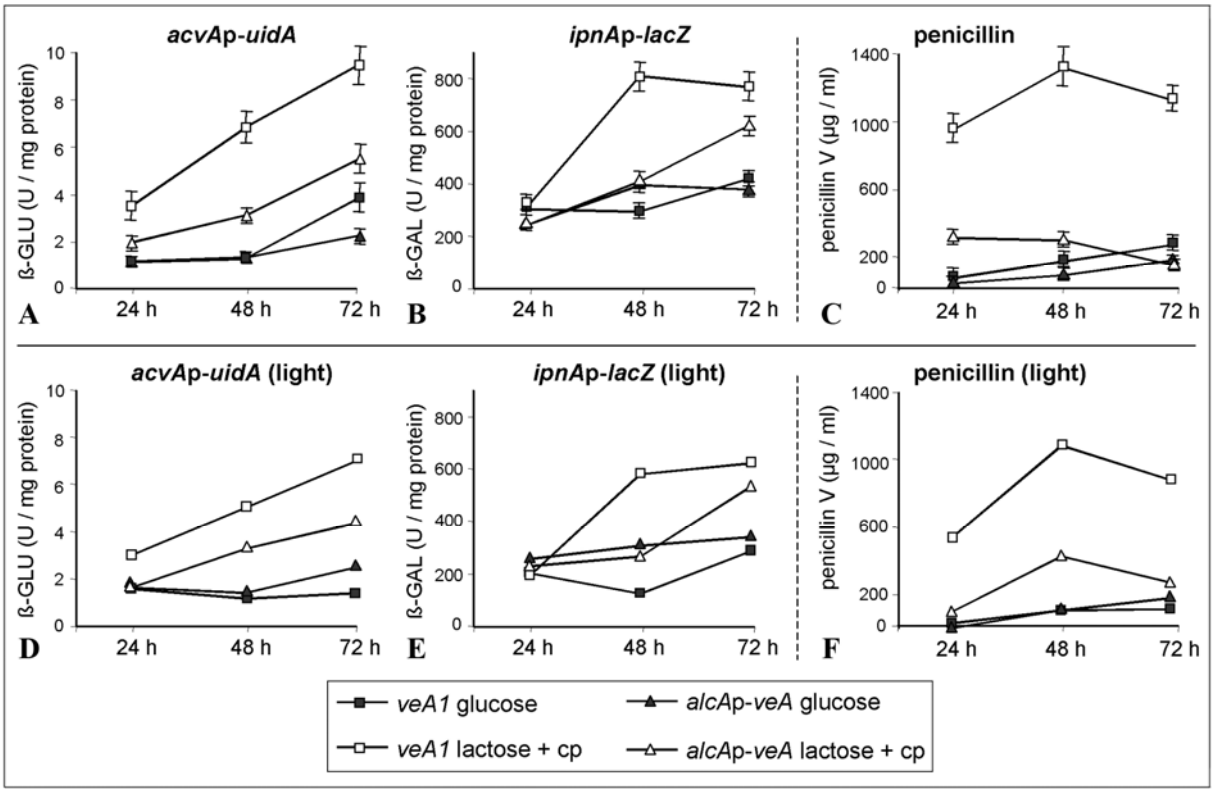

Fig. 8. Fermentation runs of *A. nidulans* strains AXB4A2 (*veA1*) and AX a-veA (*alcA*p-*veA*) with glucose (*alcA*p-repressing) or lactose + cp (*alcA*p-inducing). (**A-C**) Incubation in the dark; (**D-F**) incubation in the presence of white light. Supernatants were used for determination of penicillin production (C, F), mycelia for *acvA*p-*uidA* (A, D) and *ipnA*p-*lacZ* (B, E) fusion gene expression determined as β-GLU and β-GAL specific activity, respectively. Data of each time point represent the mean and SD of three simultaneously harvested flasks (A-C), or derive from one control experiment with white light (D-E).

Because of the medium and the longer incubation time applied, the effect of the used carbon sources on penicillin biosynthesis independent of *veA* expression has to be taken into

account when evaluating the FM data. In general, lactose is regarded as a non-repressing carbon source leading to higher expression of penicillin biosynthesis genes and increased penicillin production, whereas glucose has a repressing effect (Brakhage et al., 1992; Espeso & Peñalva, 1992). For the experiments depicted in Fig. 8, lactose (+ cyclopentanone) had the additional function of inducing *veA* expression in strain AX a-veA. Under these inducing conditions strain AX a-veA showed lower *acvA*p-*uidA* expression compared with the expression level observed in the *veA1* strain AXB4A2, whereas with glucose lower values were observed for both strains (Fig. 8A). Since the *acvA*-encoded ACVS catalyses the rate-limiting step of penicillin biosynthesis in FM (Kennedy & Turner, 1996), this finding well agrees with the here observed penicillin titres (Fig. 8C). Although with lactose as the carbon source and a thus higher penicillin titre in the untransformed *veA1* strain AXB4A2, in strain AX a-veA this effect was counteracted by induction of *veA* expression; as a consequence, penicillin titre was low under both *alcA*p-inducing and -repressing conditions.

Taken together, results from both AMM and FM indicate that expression of *veA* in *A. nidulans* leads to repression of *acvA* gene expression and to reduced penicillin titres. The effect on *ipnA*p-*lacZ* was delayed and in the end less prominent in FM (Fig. 8B); the former may be the reason why this effect was not detected in AMM after 24 h (Fig. 7B). Cultures incubated in the presence of white light showed the same tendencies for reporter-gene expression and penicillin titre, although the repressing effect of VeA seemed to be less severe (Fig. 8D-F). However, as already mentioned above, the equal availability of light for all of the mycelia was rather doubtful. On the other hand, if the amount of light that was available was sufficient to prevent nuclear localisation of parts of the overproduced VeA, this would explain the less prominent effect observed.

1.4. Control experiments

To exclude that the presence of the *veA1* allele or of the *alcA*p system influenced the results obtained so far, several control experiments were carried out.

1.4.1. Overexpression of *veA* in a *veA* deletion background

Since the repressing effect of *veA* overexpression could be demonstrated best by analysing penicillin titres in FM (Fig. 8C), this series of experiments was repeated in the *veA* deletion strain ΔveA (Table 2). Using plasmids pAL4-veA and pabaAnid providing the selectable marker (Table 3), strain ΔveA was transformed to *p*-aminobenzoic acid prototrophy. Similar to AX a-veA, a transformed strain with multiple copies of pAL4-veA

integrated into the genome was identified by Southern blot analysis (Fig. 9A) and designated ΔveA a-veA. Formation of asexual spores by this strain could be repressed under *alcA*p-inducing conditions (Fig. 9B), showing the functionality of the *alcA*p-*veA* system. This was further confirmed by the fact that the production of the dark pigment typical for Δ*veA* strains (Krappmann et al., 2005) was stopped under *alcA*p-inducing conditions in strain ΔveA a-veA.

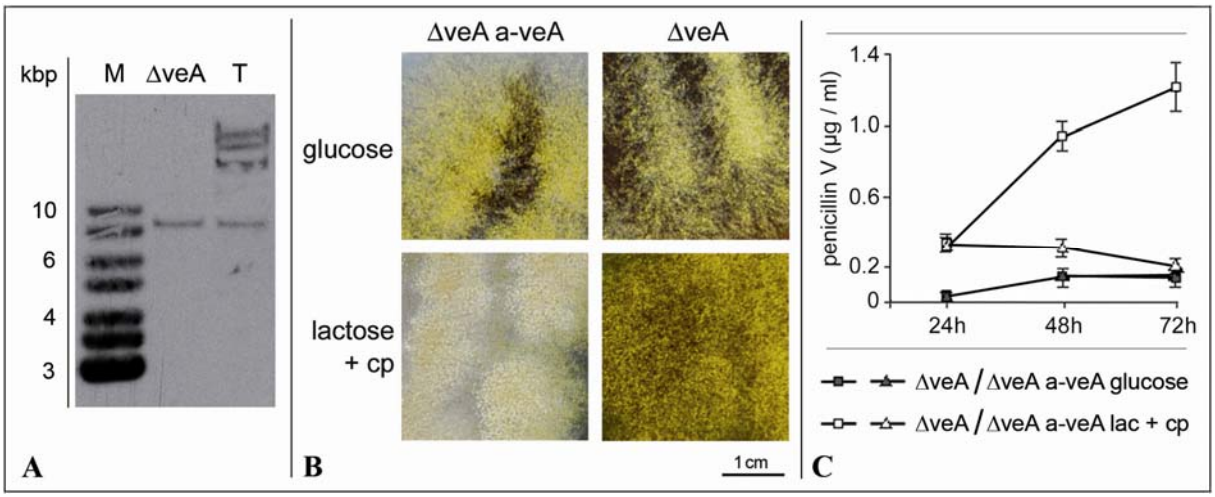

Fig. 9. Genetic and phenotypic characterisation of *A. nidulans* strains ΔveA and ΔveA a-veA. (**A**) Southern blot analysis using the probe indicated in Fig. 5A. Genomic DNA was digested with *Xba*I. Due to the *veA* deletion, the probe only detected the endogenous *alcA* promoter of strain ΔveA. Additional signals of strain ΔveA a-veA (T) indicated ectopic integrations of the *alcA*p-*veA* fusion into the genome. (**B**) Phenotypic characterisation. Upper panels: *alcA*p-repressing conditions. Lower panels: *alcA*p-inducing conditions. Growth, sporulation (yellow conidia due to *yA2* mutation) and pigment formation on AMM agar plates incubated at 37°C for 72 h in the dark. (**C**) Penicillin production of ΔveA and ΔveA a-veA. Strains were fermented with glucose (*alcA*p-repressing) or lactose plus cyclopentanone (*alcA*p-inducing) as carbon source, and supernatants were assayed. Data of each time point represent the mean and SD of three simultaneously harvested flasks.

When both, the transformed strain ΔveA a-veA and the untransformed strain ΔveA, were grown in fermentation medium either under *alcA*p-inducing or *alcA*p-repressing conditions, comparable results were obtained as with the respective *veA1* strains AX a-veA and AXB4A2 (Fig. 9C and 8C, respectively). In the untransformed Δ*veA* strain, the penicillin titre was much higher when the strains were grown with lactose plus cyclopentanone than with glucose, although the non-repressing effect of lactose on penicillin biosynthesis in general was delayed in comparison to the *veA1* strain AXB4A2. Therefore, VeA1 might play a role in this early stage of penicillin production. In the transformed strain ΔveA a-veA, however, lactose plus cyclopentanone induced *alcA*p-*veA* expression which, consequently, led to very low penicillin titres. These data indicate that irrespective of the genetic background of the strain, *veA1* or Δ*veA*, VeA acted as a repressor of penicillin biosynthesis.

1.4.2. Overexpression of *veA* in a *niiA*p-driven system

Since the nitrite reductase promoter of *A. nidulans* (*niiA*p) represents a well established inducible system (Punt et al., 1995; Kim et al., 2002), it was applied for providing an *alcA*p-independent control of inducible *veA* gene expression. The *niiA* promoter is induced by nitrate and repressed by ammonia in the medium and is thus independent of the carbon source.

Plasmid pKTB-niiAp-veA (Fig. 10A, Table 3) carries the *veA* gene under the control of the *niiA* promoter. It was generated by amplifying the 3.0 kbp *niiA*p-*veA* cassette of strain OVAR5 using oligonucleotides niiAp_KpnI and veA_3'_KpnI (Table 4). After sub-cloning, sequencing and restriction with *Kpn*I, this cassette was cloned into the *Kpn*I linearised pKTB1 vector (Table 3), which also contains the *pyr-4* gene as selectable marker. Afterwards, the *A. nidulans veA1* strain AXB4A2 was transformed to uracil prototrophy, and a transformed strain with multiple copies of pKTB-niiAp-veA integrated into the genome was identified by Southern blot analysis (Fig. 10B) and designated AX n-veA. Formation of asexual spores by this strain was reduced under *niiA*p-inducing conditions, i.e., nitrate (Fig. 10C), confirming the functionality of the *niiA*p-*veA* system.

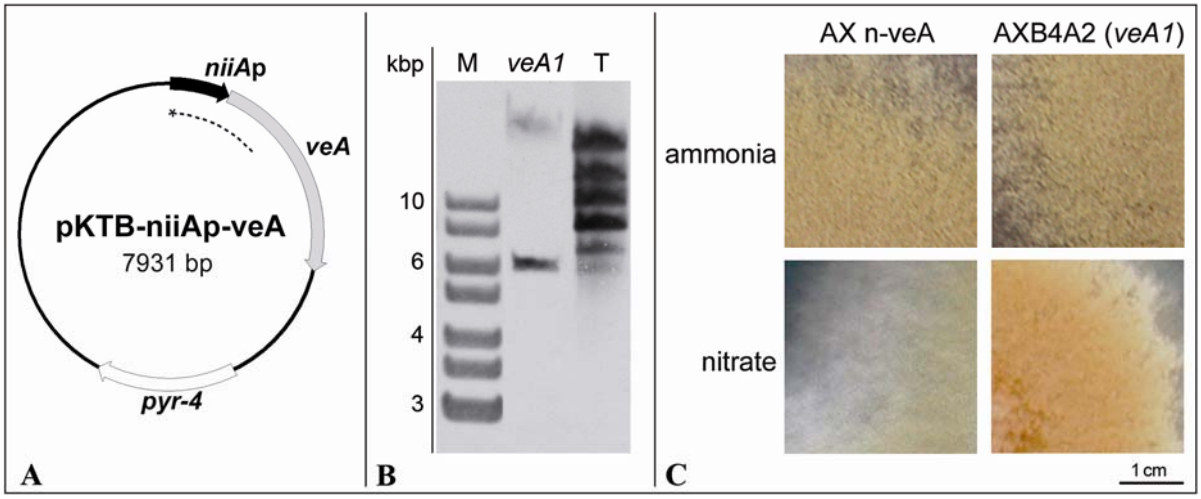

Fig. 10. Generation of *A. nidulans* strain AX n-veA and phenotypic characterisation. (**A**) Schematic map of the *niiA*p-*veA*-encoding plasmid pKTB-niiAp-veA. *pyr-4* gene from *N. crassa*. * indicates the probe used for Southern blot analysis shown in (B). It was generated by PCR amplification of a 0.8 kbp *niiA*p-*veA* fragment using the oligonucleotides niiAp-KpnI and probe_a-veA_3' (Table 4), and thus, also detected the endogenous *niiA*p and the *veA1* gene. (**B**) Southern blot analysis of the untransformed *veA1* strain AXB4A2 and AX n-veA (T) using the probe indicated in (A). Genomic DNA was digested with *Xma*I that does not cut pKTB-niiAp-veA. AX n-veA still showed wild-type bands, but additional signals were indicative of ectopic integrations of the *niiA*p-*veA* cassette. (**C**) Phenotypic characterisation. Upper panels: *niiA*p-repressing conditions [0.2 % (w/v) ammonium tartrate]. Lower panels: niiAp-inducing conditions [0.3 % (w/v) sodium nitrate]. Growth and sporulation (brown conidia due to *fwA1* mutation) on AMM agar plates incubated at 37°C for 72 h in the dark.

Both strains AXB4A2 and AX n-veA were analysed for *acvA*p-*uidA* expression and penicillin production in FM (Fig. 11), since in the *alcA*p system, VeA had most prominent effects on both aspects. Because of *niiA*p-repressing amounts of ammonia within corn steep solids, this compound was reduced to 10 % of the usual concentration in FM, which led to less penicillin production in general. For *niiA*p-repression, ammonium tartrate, and for *niiA*p-induction, sodium nitrate was added as a nitrogen source. In contrast to the carbon source, penicillin biosynthesis in *A. nidulans* was not found to be regulated by the nitrogen source (Brakhage et al., 2004). Therefore, side effects similar to those observed for the *alcA*p system could be excluded.

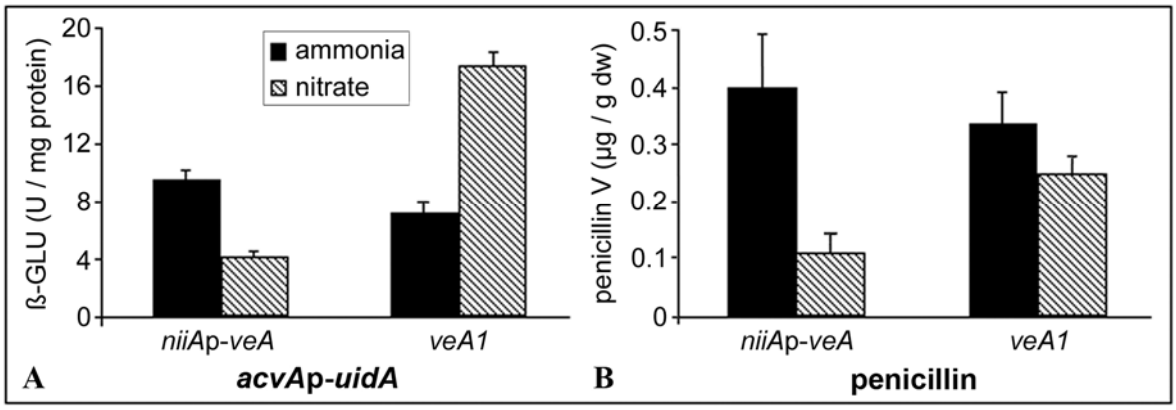

Fig. 11. Fermentation runs of *A. nidulans* strains AXB4A2 (*veA1*) and AX n-veA (*niiA*p-*veA*). Corn steep solids were reduced to 10 % of the usual concentration of FM. 0.2 % (w/v) ammonia for *niiA*p repression (■), and 0.3 % (w/v) nitrate for *niiA*p induction (☒), respectively, was added as nitrogen source. Data of each time point represent the mean and SD of three simultaneously harvested flasks. (**A**) *acvA*p-*uidA* gene expression after 72 h determined as β-GLU specific activity. (**B**) Penicillin production after 72 h.

Under inducing conditions, in the untransformed *veA1* strain AXB4A2 the *acvA*p-*uidA* expression was increased, whereas the penicillin titres of cultures grown in nitrate and ammonia were about the same (Fig. 11). The rather unexpected finding that increased *acvA* expression was not followed by increased penicillin production (compare to Fig. 8) might be explained by the modified FM. In the transformed strain AX n-veA, however, induction of *veA* expression by nitrate led to a reduced *acvA*p-*uidA* expression compared to cultures incubated with ammonia (Fig. 11A). This was also reflected by a lower penicillin titre under nitrate than under ammonia conditions (Fig. 11B). Hence, the use of the inducible *niiA* promoter to control *veA* expression suggested that VeA is a repressor of *acvA* expression and, as a consequence, of penicillin biosynthesis. Taken together, these findings confirmed the results obtained with the *alcA*p-*veA* expression cassette and thus excluded artefacts which could have resulted from the genetic system.

1.5. Analysis of a putative repressor binding site within the *acvA* promoter

Previously, a deletion analysis of the intergenic region between *acvA* and *ipnA* led to the conclusion that a specific region of the *acvA* promoter (located near to or overlapping the CCAAT box I) contains a binding site for a repressor of the *acvA* gene (Litzka et al., 1998). Deletion of this site led to a tenfold increase (or de-repression) of reporter gene expression (Then Bergh et al., 1996). Since data obtained in this work were indicative of VeA acting as a repressor of *acvA* gene expression under the applied conditions, it was conceivable that VeA either represented this particular repressor or was directly involved in the regulation of the respective protein. Therefore, strain ΔCCA-G URA (Table 2) carrying a deletion of the putative repressor binding site in front the *uidA* reporter gene (i.e., an *acvA*p[mut]-*uidA* fusion) was generated by introducing the *pyrG89* mutation into strain ΔCCA-G (Then Bergh et al., 1996) by a sexual cross. The thus provided uracil auxotrophy was used as selectable marker for subsequent transformation with pAL4-veA. Similar to AX a-veA, a transformant strain with multiple copies of pAL4-veA integrated into the genome was identified by Southern blot analysis and designated ΔCCA-G a-veA. Formation of asexual spores by this strain could be repressed under *alcA*p-inducing conditions (not shown), confirming the functionality of the *alcA*p-*veA* system. Then, the influence of VeA on *acvA* gene expression in absence of the putative repressor binding site was analysed in FM. In the untransformed strain ΔCCA-G URA, expression derived from the modified *acvA* promoter showed the expected de-repression due to lack of binding of the putative repressor. That means, glucose and lactose had the same effects as in a strain without the mutated *acvA*p (e.g., AXB4A2), but on a tenfold increased level (not shown). However, overexpression of *veA* in strain ΔCCA-G a-veA still led to reduction of *acvA*p-*uidA* gene expression compared to *alcA*p-repressing conditions, making it unlikely that VeA mediates this effect *via* the deleted *cis*-acting site. Otherwise, no or only little differences to the untransformed strain ΔCCA-G URA would have been observed. Hence, there must be another, VeA-independent repressor of the *acvA* gene.

2. AnBH1 and PkcA

2.1. AnBH1 and phosphorylation

Whereas the exact mechanism of repression of *acvA* gene expression by VeA remains to be elucidated, the mode of repression of the *aatA* gene by the *A. nidulans* basic-region helix-loop-helix (bHLH) protein 1 (AnBH1) was shown by Caruso et al. (2002). It directly binds as a homodimer to an asymmetric E-box within the *aatA* promoter and thus counteracts the positive action of the CCAAT-binding factor AnCF. On the other hand, while external signals regulating VeA are already known (e.g., light), there is only little information available on signals and their transduction cascades leading to AnBH1. Because all bHLH transcription factors were shown to be phosphorylated (Littlewood & Evan, 1994), it was likely that phosphorylation might also play a role in regulation of AnBH1.

To investigate whether AnBH1 can be phosphorylated in general, the in *E. coli* overproduced protein was purified as MalE fusion (Caruso et al., 2002), cleaved from MalE with factor Xa protease and treated with λ protein phosphatase (λ-PPase) to remove phosphate groups added by the heterologous host to serine, tyrosine and threonine residues. Since a removal of these negatively charged groups led to a more alkaline isoelectric point of AnBH1, this shift should be detectable by two-dimensional gel electrophoresis (2D-GE; Fig. 12).

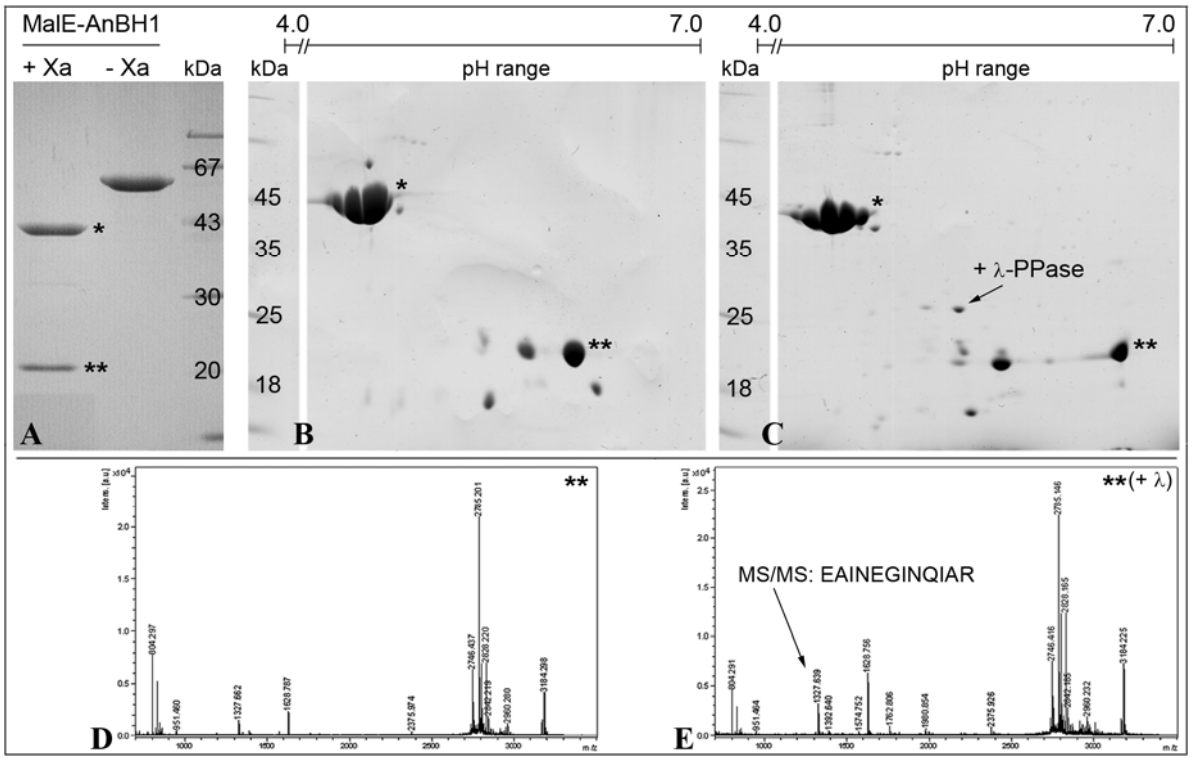

Fig. 12. 2D-GE analysis of AnBH1. (**A**) SDS-PAGE of *E. coli*-overproduced MalE-AnBH1, without (-) and with (+) factor Xa cleavage yielding MalE (*) and AnBH1 (**). (**B**) 2D-GE analyses of the cleaved, untreated

mixture. MalE (calculated molecular mass: 42 kDa, theoretical isoelectric point: pI 5.06) and AnBH1 (16 kDa, pI 6.44) are indicated. (**C**) 2D-GE analysis of the cleaved, λ-PPase-treated mixture. λ-PPase (25 kDa, pI 5.6), MalE and AnBH1 are indicated. Since AnBH1 had shifted to a more alkaline position that is closer to the theoretical pI of 6.44 that is characteristic for the unmodified protein, a dephosphorylation event was likely. (**D+E**) For identification of AnBH1, the respective spots were excised. Proteins were eluted and subjected to tryptic digestion followed by mass spectrometry (MS) analysis: mass spectrum before (D) and after (E) λ-PPase treatment. The indicated signal was assigned by MS/MS analysis to amino acids 29 till 40 of AnBH1.

When the λ-PPase-treated mixture was separated on a two-dimensional gel (Fig. 12C), the spot representing AnBH1 (verified by MS/MS analysis, Fig. 12E) appeared – compared to AnBH1 of the untreated mixture (Fig. 12B+D) – at a pH value closer to that one predicted for the isoelectric point of the unmodified protein (pI 6.44). The untreated protein was detected in a more acidic region indicating more negative charges, likely due to posttranslationally added phosphate groups that could be removed by λ-PPase-treatment. It has been known for a while that *E. coli* possesses eukaryotic-like serine / threonine protein kinases (Kennelly, 2002). Therefore, although in this experiment performed by the prokaryotic host, it was conceivable that serine and threonine residues – amino acids that are often phosphorylated in eukaryotic signal transduction pathways (Santos & Shiozaki, 2001) – are also putative targets of phosphorylation of AnBH1 in *A. nidulans*.

To get a hint which residues of AnBH1 are likely to be phosphorylated, its amino acid sequence was analysed by the NetPhos2.0 software (www.cbs.dtu.dk/services/NetPhos). Since the third annotation of the *A. nidulans* genome was not completed yet, the published 139 amino acid version of the protein (Caruso et al., 2002), that was also used for the 2D analysis in Fig. 12, was subjected to the prediction. As depicted in Fig. 13, Ser^{9}, Ser^{71} and Tyr^{123} were putative candidates, with Ser^{71} being most promising, since it was part of a conserved protein kinase C (PKC) target sequence (J. Heinisch, personal communication).

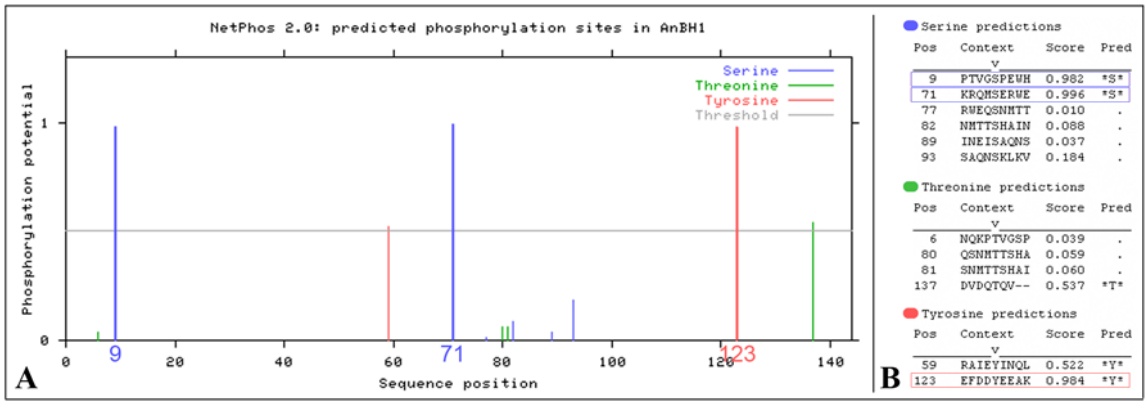

Fig. 13. NetPhos 2.0 prediction of serine, threonine and tyrosine phosphorylation sites in AnBH1. Schematic view (**A**) and score table (**B**), modified from the output file. The threshold was set to 0.500 by the

programme; output scores below or just barely above indicated a quite low confidence for this site being a true phosphorylation site. The most prominent sites Ser^{9}, Ser^{71} and Tyr^{123} are also highlighted in the table.

2.2. Regulation of subcellular localisation of AnBH1 by a PKC

Due to this conserved PKC target sequence -**R**QM**S**^{71}E**R**- of AnBH1, an involvement of a protein kinase C in the regulation of the transcription factor and, consequently, in penicillin biosynthesis was conceivable. Many, if not all, of these serine / threonine kinases are central components of signal transduction chains that are, in part, already well investigated (Schmitz & Heinisch, 2003). Thus, a participation of a PKC in the regulation of a penicillin biosynthesis regulator would open the door to further upstream parts of the signalling cascade and probably also to new external triggers of penicillin biosynthesis. Since with calphostin C a potent and specific inhibitor of PKC was available (Kobayashi et al., 1989), PKC-dependent cellular localisation of (the 139 amino acid) AnBH1 could be analysed in *A. nidulans* strain LOGOAnBH1 that contains an *anbH1-egfp* gene fusion integrated into the genome (Table 2). The strain was cultivated in AMM either with 5 µM (final concentration) or without calphostin C, and in the presence of light since this is needed for full activation of the inhibitor (Bruns et al., 1991). Results are shown in Fig. 14.

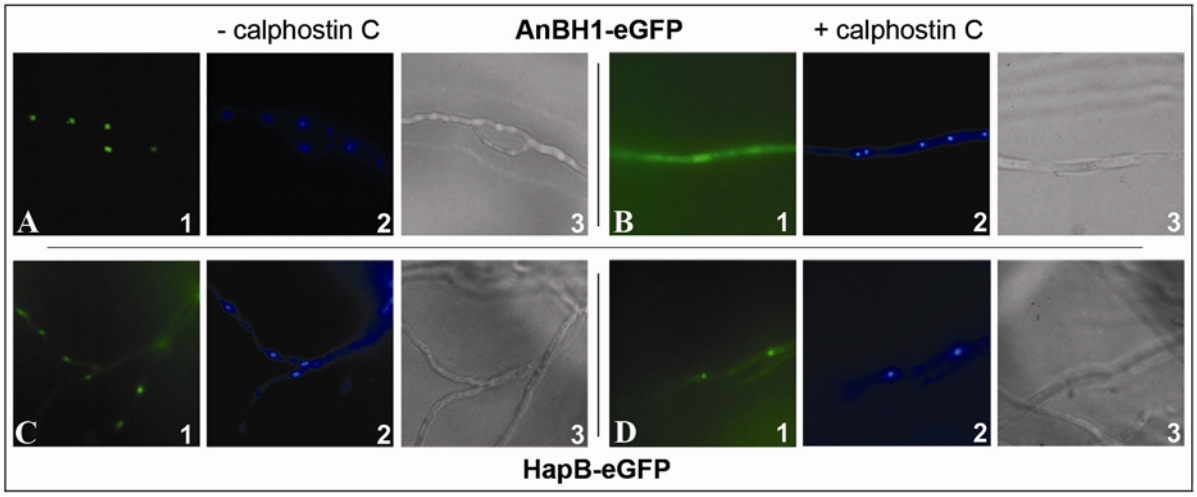

Fig. 14. Cellular localisation of AnBH1-eGFP and HapB-eGFP fusion proteins, depending on the presence of the PKC inhibitor calphostin C. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; panels 2). Samples were analysed by fluorescence microscopy (panels 1 showing localisation of the fusion proteins, and panels 2 showing nuclei) or light microscopy (panels 3). (**A**+**B**) AnBH1-eGFP-derived fluorescence of *A. nidulans* strain LOGOAnBH1 without (A) and after addition (B) of calphostin C. (**C**+**D**) HapB-eGFP-derived fluorescence of *A. nidulans* strain HapB-eGFP without (C) and after addition (D) of the PKC inhibitor.

Without calphostin C, the AnBH1-eGFP fusion protein was found to be located in the nucleus (Fig. 14A). By contrast, addition of the PKC inhibitor led to a decrease in the

fluorescence in the nucleus, and additionally, fluorescence was visible in the cytoplasm (Fig. 14B), suggesting that AnBH1-eGFP either could not enter the nucleus any more or was actively exported from that organelle. As a control, the nuclear localisation of a HapB-eGFP fusion was studied. HapB does not contain such obvious PKC phosphorylation sites. As shown in Figs. 14C and D, the fusion protein of *A. nidulans* strain HapB-eGFP (Table 2) was located in the nucleus irrespective of whether calphostin C was added to the culture medium. It was thus likely that a PKC influenced the nuclear localisation of AnBH1.

While the experiments for this work have been performed, colleagues identified two putative PKC-encoding genes in *A. nidulans* designated *pkcA* and *pkcB*, with the encoded PkcA showing more similarities to typical PKCs than PkcB (see Herrmann et al., 2006, that also includes data of this work). Hence, it was more likely that PkcA was involved in phosphorylation and thus regulation of nuclear localisation of AnBH1, because calphostin C interacts with the regulatory domain which is conserved in PkcA but not in PkcB. To confirm this assumption, localisation of the AnBH1-eGFP fusion protein was analysed in the *A. nidulans* strain PkcAinv1.3 (Table 2) that encodes an inducible *alcA*p-*pkcA*^{antisense} construct to knock down *pkcA* gene expression. A deletion of the gene was not successful, most likely because PkcA is an essential enzyme. Strain PkcAinv1.3 was shown to have elevated levels of *aatA*p-*lacZ* reporter gene expression under *pkcA*^{antisense}-inducing conditions, i.e., in the absence of PkcA, indicating a positive effect of PkcA on nuclear localisation of AnBH1 as repressor of *aatA* gene expression (Herrmann et al., 2006). Consequently, under these *alcA*p-inducing conditions the AnBH1-eGFP fusion protein was expected to be located, at least in part, in the cytoplasm. To prove this hypothesis, strain PkcAinv1.3 was cultivated in AMM under either *alcA*p-*pkcA*^{antisense}-repressing or -inducing conditions, and localisation of AnBH1-eGFP was followed. Results are shown in Fig. 15.

Under *alcA*p-*pkcA*^{antisense} repressing conditions (glucose), the AnBH1-eGFP fusion protein of strain PkcAinv1.3 was found to be located in the nucleus (Fig. 15A). By contrast, when *pkcA*^{antisense} expression was induced by lactose plus cyclopentanone (cp), fluorescence was also visible in the cytoplasm (Fig. 15B). Nevertheless, even under inducing conditions, the nuclei still showed fluorescence, as indicated by arrows in Fig. 15B1, suggesting that AnBH1-eGFP only partially entered the nucleus when *pkcA* mRNA was silenced. As a control, the AnBH1-eGFP fusion protein was analysed in strain C2.3 which does not contain the *alcA*p-*pkcA*^{antisense} construct (Table 2). As shown in Figs. 15C and D, the AnBH1-eGFP fusion protein was located in the nucleus irrespective of incubation with glucose or with lactose plus cyclopentanone. Hence, the different carbon sources alone had no influence on

localisation of AnBH1. These results indicated that reduced expression of *pkcA* due to production of *pkcA* antisense RNA led to reduced localisation of AnBH1 in the nucleus and, furthermore, to an accumulation of the protein in the cytoplasm.

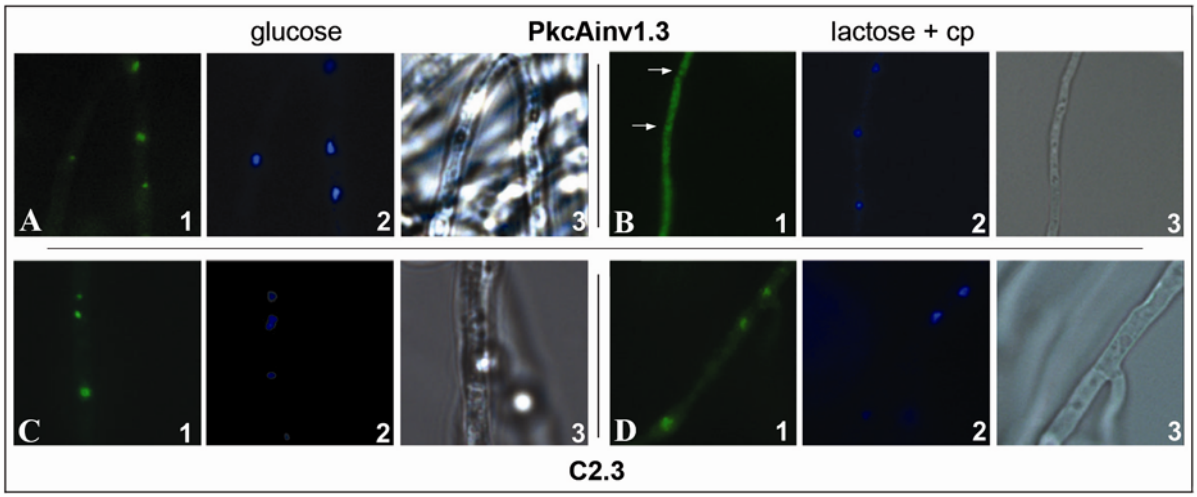

Fig. 15. Cellular localisation of the AnBH1-eGFP fusion protein in strains PkcAinv1.3 and C2.3, depending on *alcA*p induction. Nuclei were stained with DAPI (panel 2). Samples were analysed by fluorescence microscopy (panel 1 showing localisation of the fusion protein; and panel 2 showing nuclei) or light microscopy (panel 3). (**A**+**B**) AnBH1-eGFP-derived fluorescence of *A. nidulans* strain PkcAinv1.3 under *alcA*p-repressing (A) and *alcA*p-inducing conditions (B). Arrows in B1 indicate nuclei. (**C**+**D**) AnBH1-eGFP-derived fluorescence of *A. nidulans* control strain C2.3 when incubated with glucose (C) and lactose + cp (D).

2.3. The role of the putative phosphorylation site Ser^{71} of AnBH1

Data obtained so far suggested that PkcA regulates nuclear localisation of AnBH1 and, as a consequence, *aatA* gene expression. The protein thus represents another part of the complex network regulating penicillin biosynthesis. However, the question remained whether PkcA uses the predicted motif surrounding residue Ser^{71} of AnBH1 for direct phosphorylation, or whether regulation occurs *via* a cascade with intermediate kinases using different target amino acid residues of AnBH1. Such a cascade could involve a classical MAP kinase pathway that often acts downstream of a PKC (Heinisch, 2005). Direct phosphorylation of a transcription factor by PKC has been described for the *Neurospora crassa* protein WC-1 (Arpaia et al., 1999; Franchi et al., 2005; Loros, 2005).

2.3.1. Analysis of amino acid exchanges at position 71 of AnBH1-eGFP

To analyse whether Ser^{71} is important for the nuclear localisation of AnBH1, the residue was mutated to an alanine (S71A) and to a glutamic acid residue (S71E), respectively. Alanine is a non-polar amino acid and can not be phosphorylated, whereas glutamate due to

its negative charge mimics phosphorylation. Thus, S71A represented a non-phosphorylated, and S71E a permanently phosphorylated version of the amino acid. Using eGFP fusion proteins, the impact of a (non-) phosphorylated Ser^{71} could be analysed microscopically.

For generation of plasmid p123-anbH1-S71A carrying the *anbH1*(S71A)-*egfp* gene fusion (Table 3), the PCR method of Higuchi (Higuchi et al., 1988) was applied to introduce the respective codon exchange (Fig. 16B) using oligonucleotides anbH1-gfp/Bam, anbH1-Ala1, anbH1-Ala2 and anbH1-gfp/Nco (Table 4). After sub-cloning and sequencing of the thus generated *anbH1*(S71A) fragment, it was ligated into the *BamH*I/*Nco*I-linearised p123 vector to generate the *egfp* fusion. Afterwards, the *pabaA* gene of plasmid pabaAnid (Table 3) was cut by *Kpn*I restriction and introduced as a selectable marker for *A. nidulans* to yield p123-anbH1-S71A. The same *pabaA* fragment was ligated into the *Kpn*I-linearised plasmid pLOGO201E carrying the *anbH1*(S71E)-*egfp* gene fusion (Martic, 1999) to yield p123-anbH1-S71E (Table 3). Mutations were confirmed by sequencing (Fig. 16A+B).

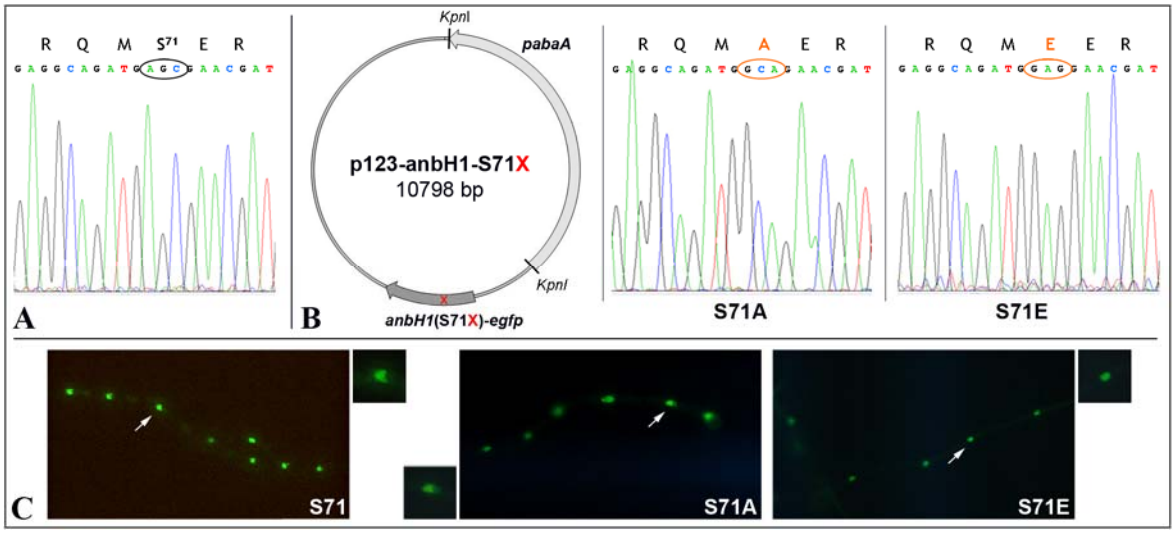

Fig. 16. Amino acid exchanges at position 71 of AnBH1 in eGFP fusion proteins. (**A**) Chromatogram of the *anbH1* wild-type sequence encoding the putative PKC target sequence RQMSER with the central Ser^{71} residue. (**B**) General map of the plasmids encoding the modified AnBH1-eGFP variants (X means alanine or glutamic acid); and chromatograms of the respective S71A and S71E exchanges. (**C**) Fluorescence-microscopic analysis of the localisation of the different AnBH1-eGFP fusion proteins. S71: LOGOAnBH1 (no modification). S71A: AnBH1(S71A)-GFP. S71E: AnBH1(S71E)-GFP. Indicated nuclei are also shown with a higher magnification.

Using plasmids p123-anbH1-S71A and p123-anbH1-S71E, *A. nidulans* strain R21 was transformed to *p*-aminobenzoic acid prototrophy, thus generating strains AnBH1(S71A)-GFP and AnBH1(S71E)-GFP, respectively (Table 2). Afterwards, localisation of the eGFP fusion proteins in both strains was analysed and compared to localisation of the wild-type protein fusion in strain LOGOAnBH1 (Fig. 16C). Surprisingly, the S71A-mutant protein was still

located mainly in the nucleus, as was – as expected – the wild-type and the S71E-mutant protein. Thus, it appeared that phosphorylation of Ser^{71} (or the possibility to phosphorylate this residue) is not essential for nuclear localisation of AnBH1. However, a higher magnification of the nuclei (small pictures in Fig. 16C) showed that fluorescence of the S71E-mutated protein was more compact and much denser than that of the wild-type protein with an unknown phosphorylation level and of the S71A-mutant protein which can not be phosphorylated at position 71. This implies that phosphorylation of Ser^{71} supports nuclear transport of AnBH1 but is not absolutely necessary, and that the Ser^{71} residue of the wild-type protein is not or not completely phosphorylated under the applied conditions.

Since AnBH1 was shown to form homodimers (Caruso et al., 2002), it was necessary to rule out the possibility that the AnBH1(S71A)-eGFP fusions enter the nucleus after dimerisation with the wild-type AnBH1 proteins that were not eGFP-tagged but still present in the background of strain AnBH1(S71A)-GFP. Therefore, *A. nidulans* strain 11.4 (Table 2) was transformed using plasmid p123-anbH1-S71A to yield strain 11.4-AnBH1(S71A). In this strain the *anbH1* gene was under the control of the inducible *alcA* promoter (Table 2), and thus, could be silenced by glucose. Under these *alcA*p-repressing conditions, i.e. without or with only marginal presence of a wild-type dimerisation partner, the AnBH1(S71A)-eGFP fusion protein was still located mainly in the nucleus, as it was under *alcA*p-inducing conditions (Fig. 17). Hence, this experiment confirms that nuclear localisation of AnBH1 is independent of phosphorylation of Ser^{71}. However, association of the AnBH1(S71A)-eGFP fusion protein with another bHLH transcription factor, followed by nuclear import of this heterodimer can not be excluded, although other dimerisation partners of AnBH1 are not known yet.

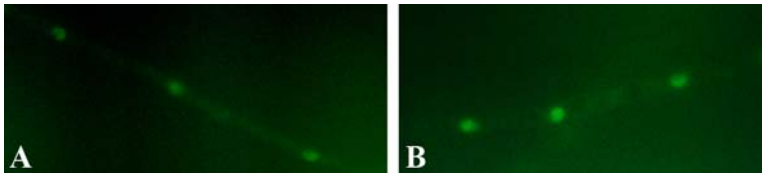

Fig. 17. Cellular localisation of the AnBH1(S71A)-eGFP fusion protein in strain 11.4-AnBH1(S71A) depending on *alcA*p-*anbH1* induction. (**A**) *alcA*p-inducing conditions, i.e., in presence of wild-type AnBH1. (**B**) *alcA*p-repressing conditions, i.e., without wild-type AnBH1 as potential dimerisation partner.

2.3.2. Analysis of the DNA-binding capability of AnBH1(S71A)

Data obtained so far showed that Ser^{71} is not exclusively responsible for nuclear localisation of AnBH1 and that its phosphorylation at most has a supporting effect on this. Thus, there have to be other PkcA-involving regulatory mechanisms – otherwise, inhibition of

this kinase would not have led to cytoplasmic localisation of AnBH1 (Figs. 14B and 15B). It was shown for other transcription factors that it is often not the phosphorylation of only a single amino acid by which nuclear localisation is regulated (Komeili & O'Shea, 1999; Beals et al., 1997; Biggs et al., 1999) – and that other modifications like acetylation can also play a role for bHLH proteins (Faiola et al., 2007). In addition, phosphorylation of transcription factors not necessarily has to be involved in regulation of nuclear entry, but might also affect protein stability, interaction with co-regulators and DNA binding (Whitmarsh & Davis, 2000). Although the latter is a wide-spread phenomenon that was also described for bHLH proteins (Berberich & Cole, 1992; Boyle et al., 1991), it was rather unlikely that phosphorylation of Ser^{71} directly influenced binding of AnBH1 to target gene promoters, because it is not part of the bHLH motif spanning from amino acid residues 16 to 64 (Caruso et al., 2002). Therefore, it is most likely not involved in DNA binding and its prerequisite, the dimerisation *via* the HLH domain (Lassar et al., 1991; Massari & Murre, 2000).

To confirm this assumption, an S71A-mutated variant of AnBH1 was heterologously produced in *E. coli* as a MalE fusion, similar to the wild-type MalE fusion used for the initial experiments (Fig. 12; Caruso et al., 2002). To introduce the Ser to Ala mutation, Higuchi PCR was performed using oligonucleotide pairs S71A_BamHI-5' and anbH1-Ala1, as well as anbH1-Ala2 and S71A_SalI-3' (Table 4), and pMAL-anBH1 (Table 3) as the template. The final PCR product was sub-cloned, sequenced and ligated *via* the *Bam*HI/*Sal*I restriction sites into the pMAL-c2X expression vector to give plasmid pMAL-anBH1-S71A, which was used to transform *E. coli* DH5α. After induction of *malE-anBH1*(S71A) gene expression with 0.1 mM IPTG, the MalE-AnbH1(S71A) fusion protein was purified *via* amylose-affinity chromatography. Then, it was analysed for *in vitro* binding to the *aatA*-promoter fragment that was previously shown to be bound by the recombinant wild-type protein (Caruso et al., 2002). This 50 bp DNA probe contained the E-box as AnBH1 binding motif and was generated by hybridising oligonucleotides Shift1 and Shift2 (Table 5).

Fig. 18 shows the result of the electrophoretic mobility shift assay (EMSA) of (factor Xa cleaved) MalE-AnBH1(S71A) compared to the wild-type (MalE-)AnBH1. Binding of the proteins to the labelled DNA altered the migration of the probe resulting in a shift that depended on the size of the complex – a complex of DNA and MalE-fused AnBH1 (e.g., (1) in Fig. 18) led to a stronger retardation than one including the free AnBH1 protein (2). Since addition of both proteins, AnBH1 and AnBH1(S71A), retarded the migration of the probe to a similar extend, both were able to form a complex with the labelled DNA. Thus, the S71A mutation did not alter the binding (or the preceded dimerisation) of recombinant AnBH1 to

the *aatA*-promoter fragment. As mentioned above, this was not surprising, since the amino acid residue is not located within the bHLH motif. However, phosphorylation of Ser^{71} could have had an indirect influence, e.g., by inducing conformational changes that (un)masks the binding surface (Whitmarsh & Davis, 2000).

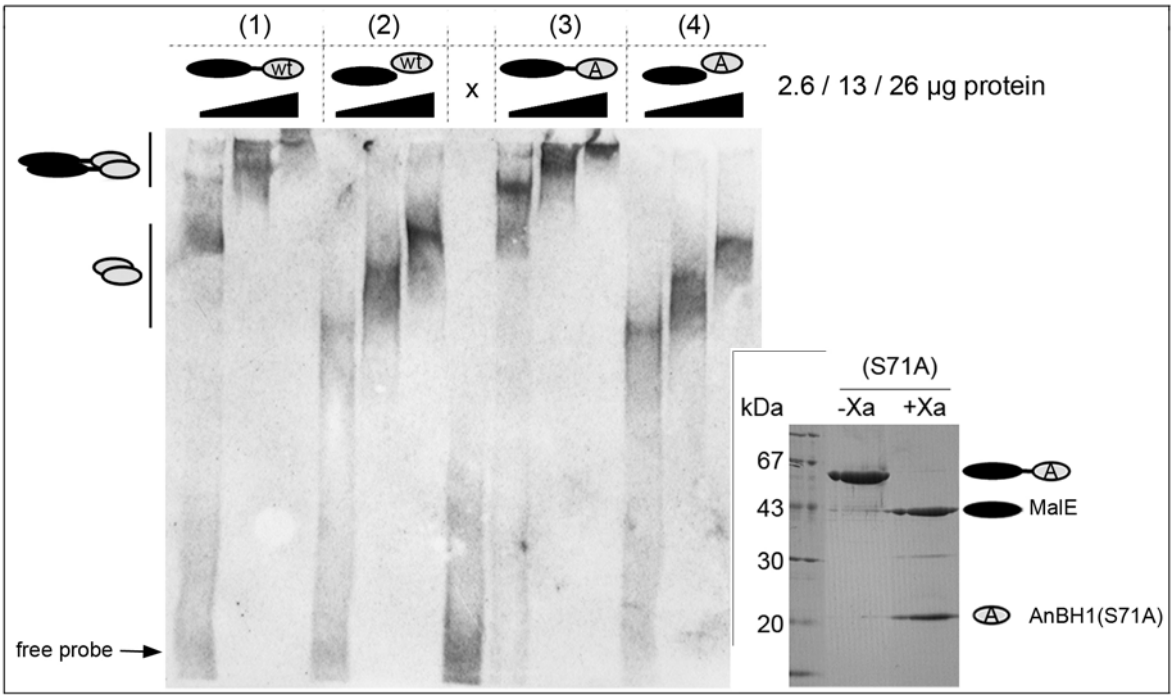

Fig. 18. *In vitro* binding (EMSA) of AnBH1 and AnBH1(S71A) to an *aatA*-promoter fragment. The SDS-PAGE on the right shows the purified MalE-AnBH1(S71A) fusion protein (before and after factor Xa cleavage), as well as the symbols used for the different proteins. EMSA (7 % (w/v) polyacrylamide gel): Both fusion proteins, MalE-AnBH1 (1+2) and MalE-AnBH1(S71A) (3+4), were assayed in increasing concentrations (2.6 – 26 µg), both in full length (1+3) and factor Xa treated (2+4). X means only DNA probe without protein. Positions of the free DNA probe and the different DNA-protein complexes are indicated on the left.

Although it is not clear whether the Ser^{71} residue of AnBH1 was phosphorylated by the heterologous *E. coli* host at all (the shift caused by the λ-PPase treatment in Fig. 12 could have resulted from other phosphate groups), data indicated that this residue does not play the important role one would expect from its location within a conserved PKC target site. It is thus more likely that PkcA exerts its effect on AnBH1 in an indirect way probably leading to phosphorylation of other residues (Fig. 13) – or that phosphorylation of Ser^{71} (either directly or indirectly) is not the crucial but rather an ancillary step in regulation of the protein. Nevertheless, the involvement of a PKC in regulation of AnBH1 may result in the discovery of novel, so far not considered external signals – e.g., cell wall stress – influencing penicillin biosynthesis (see discussion).

3. IAT and peroxisomes

3.1. Knowledge from *Penicillium chrysogenum* – also true for *Aspergillus nidulans*?

The network that regulates penicillin biosynthesis in *A. nidulans* has been continuously investigated. However, this does not fully apply to the study of the structural genes and their encoded enzymes, and to the compartmentalisation of the whole pathway in this penicillin producer. In recent years, most of the studies concerning these issues have been carried out in *P. chrysogenum*, the organism that is used for industrial penicillin production. Fig. 19 shows the sub-cellular location of the key enzymes of the pathway as it was elucidated for *P. chrysogenum* (Müller et al., 1991; Evers et al., 2004). The first two steps, involving ACVS and IPNS, are performed – likely also in *A. nidulans* (van der Lende et al., 2002b) – in the cytosol, whereas exchange of the side chain and most probably activation of the side-chain precursors take place in peroxisomes (for pathway see Fig. 2). The exact reason for the final step being separated from the cytoplasm is not clear, but it has been hypothesised that the peroxisomal lumen provides a better environment for the involved enzymes with respect to the slightly alkaline pH (van der Lende et al., 2002a; van Roermund et al., 2004), to the higher metabolic concentration of enzymes and substrates, and to aspects of regulation. One could also speculate whether a more hydrophobic milieu within the organelles, e.g., needed for fatty acid metabolism, could facilitate the generation of hydrophobic penicillins.

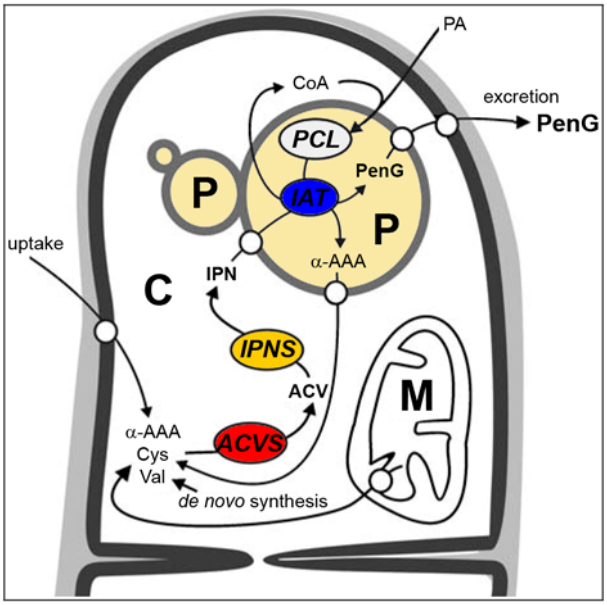

Fig. 19. Location of penicillin biosynthesis in *P. chrysogenum* (adapted from Evers et al., 2004). The three main enzymes are coloured; i.e., ACVS, IPNS and IAT. PCL, phenylacetyl (PA)-CoA ligase, presumably needed for side-chain activation. Cellular compartments: C, cytosol. M, mitochondria. P, peroxisomes. The precursor amino acids are acquired either by uptake, recycling (α-AAA) or *de novo* synthesis that partially takes place in M. The membrane barriers that the enzymes, precursors, intermediates and end-products are confronted with during biosynthesis are indicated by open circles.

Special interest has focussed on the last gene / enzyme of the penicillin biosynthesis pathway – the *aatA* gene encoding isopenicillin N acyltransferase (IAT) – because this is the pathway specific one (Figs. 2 and 3). As outlined in the introduction and depicted in Fig. 20,

the IAT of both *A. nidulans* and *P. chrysogenum* share a high sequence similarity, but in particular the role of the obviously different C-terminal tripeptide has only been studied in *P. chrysogenum.* There, this tripeptide functions as peroxisomal targeting signal 1 (PTS1) that is essential for localisation within these organelles and thus for penicillin production in general (Müller et al., 1992). For *A. nidulans*, however, peroxisomal localisation of the IAT was simply supposed without any real proof (e.g., Lutz et al., 2005), although its C-terminus would be a rather unusual PTS1. Moreover, consequences of a possible mislocalisation of the protein, e.g., for penicillin production, have not been analysed at all.

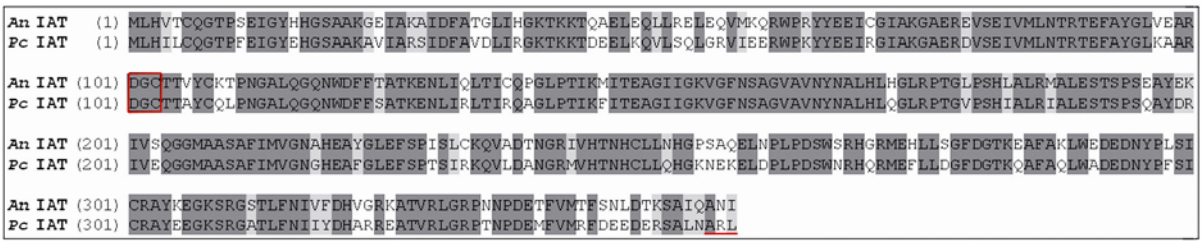

Fig. 20. Amino acid sequence alignment of *A. nidulans* (*An*) and *P. chrysogenum* (*Pc*) IAT. Identical amino acids are shaded in dark, similar amino acids in light grey, respectively. The proteins show 76% sequence identity. In *P. chrysogenum*, the active form of the enzyme results from cleavage within the boxed DGC motif, which is conserved but not utilised in *A. nidulans* (Fernández et al., 2003). One of the structural differences consists in the C-terminus that only in *P. chrysogenum* contains a typical PTS1 (underlined).

The study of Fernández et al. (2003), suggesting that both proteins, the *A. nidulans* and *P. chrysogenum* IAT, differ in their ability of being processed, clearly demonstrated that despite the high sequence identity not all characteristics might be one-to-one transferable. Hence, whereas for *P. chrysogenum* the importance of the IAT and its localisation within functional peroxisomes has been already demonstrated, the contribution of these organelles to penicillin biosynthesis in *A. nidulans* has not been fully elucidated yet.

3.2. Localisation of IAT in *A. nidulans*

A conserved PTS1 comprises three C-terminal amino acids of the form S/A R/K L/M (Swinkels et al., 1992), although some peroxisomal proteins have a cryptic or unusual PTS1 (e.g., Klein et al., 2002). It was also suggested that the context of the last 10-12 amino acids before the C-terminal end affects targeting (Brocard & Hartig, 2006). To investigate the sub-cellular localisation of the *A. nidulans* IAT and the role of its C-terminal PTS1-related tripeptide -ANI, N-terminal eGFP fusions of both the wild-type IAT and the IAT lacking the three C-terminal amino acids were analysed in different genetic backgrounds.

3.2.1. Generation of eGFP-IAT encoding plasmids

To generate plasmid pALX-aatA encoding eGFP-IAT (Table 3; Fig. 21A), the full-length *aatA* gene including terminator sequences was amplified from chromosomal DNA using oligonucleotides aatA_up_BglII and aatA_down (Table 4). After sub-cloning and sequencing, the 1,607 bp *aatA* fragment was ligated *via* the *Bgl*II and *Xba*I restriction sites into the accordingly linearised vector pALX-213 (Table 3), and pALX-aatA was yielded. To delete the putative PTS1-encoding sequence, the alanine residue within this sequence was replaced by a stop codon (Fig. 21B). Therefore, inverse PCR was performed using oligonucleotides aatA_invPCRup and aatA_invPCRdelPTS (Table 4), and pALX-aatA as template. By re-ligation of the PCR product, plasmid pALX-aatA$^{\Delta PTS1}$ encoding eGFP-IAT$^{\Delta PTS1}$ was generated (Table 3). Expression of the *egfp* gene fusions was driven by the constitutively active *gpdA* promoter of *A. nidulans* (Punt et al., 1990).

3.2.2. Localisation of IAT in a wild-type background

To investigate the cellular localisation of both eGFP-IAT and eGFP-IAT$^{\Delta PTS1}$ protein fusions in a wild-type background, *A. nidulans* strain A234 (Table 2) was transformed using plasmids pALX-aatA and pALX-aatA$^{\Delta PTS1}$, respectively. The *p*-aminobenzoic acid auxotrophy (Paba$^-$) was complemented by plasmid pabaAnid as a co-transforming agent. A certain percentage (about 20%) of the Paba$^+$ transformants also carried the *egfp* gene fusions in multiple copies (data not shown). One of each was chosen and designated GFP-IAT and GFP-IAT$^{\Delta PTS1}$, respectively. They were afterwards subjected to fluorescence microscopy.

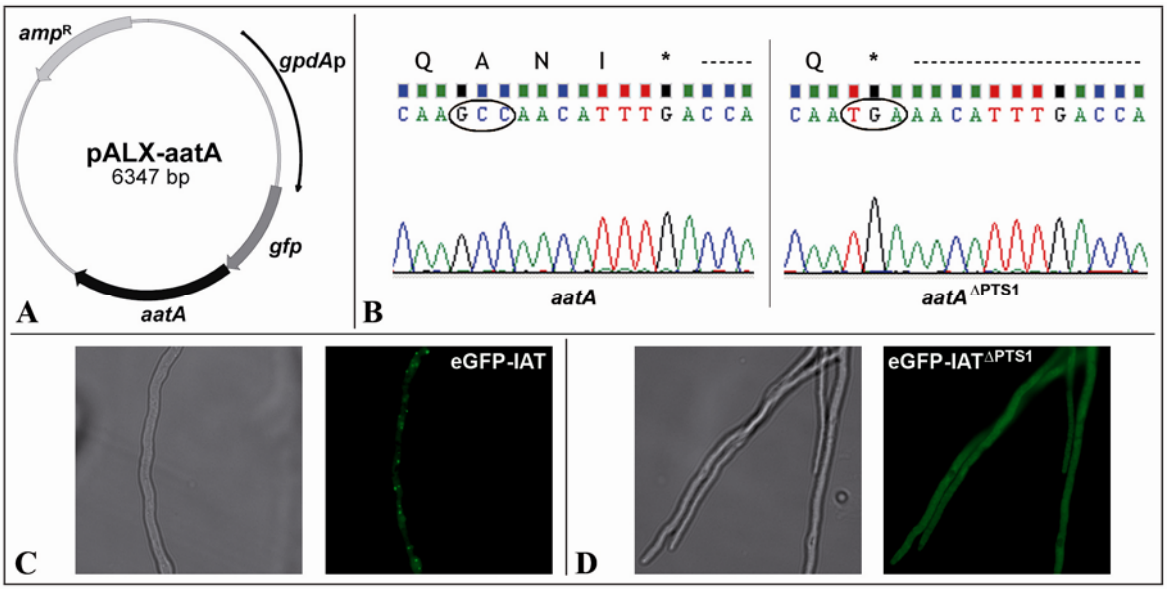

Fig. 21. Cellular localisation of *A. nidulans* IAT in a wild-type background. (**A**) Schematic map of plasmid pALX-aatA encoding the eGFP-IAT fusion protein. (**B**) Chromatograms of the *aatA* wild-type sequence

encoding the putative PTS1 (left panel), and of the nonsense Ala-to-STOP(*)-mutation resulting in deletion of this sequence in pALX-aatA$^{\Delta PTS1}$ (right panel). (**C**) Localisation of eGFP-IAT. Strain GFP-IAT was analysed by light (left panel) and fluorescence (right panel) microscopy. The punctuate dots of the latter have previously been assigned to peroxisomal localisation (see text). (**D**) Mislocalisation of eGFP-IAT$^{\Delta PTS1}$ as analysed by light (left panel) and fluorescence (right panel) microscopy of strain GFP-IAT$^{\Delta PTS1}$.

As shown in Fig. 21C, localisation of eGFP-IAT was mainly restricted to punctate dots that have been shown previously to be microbodies (e.g., Szewczyk et al., 2001, and Maggio-Hall & Keller, 2004, for the peroxisomal proteins AcuD and AcuE, respectively). It was thus very likely that the *A. nidulans* IAT was also located within these organelles (which could be confirmed by the analysis of peroxin mutant strains; see below). However, the well-defined pattern was lost for the eGFP- IAT$^{\Delta PTS1}$ fusion protein in which the putative PTS1 was deleted. The localisation of this protein was mainly cytosolic (Fig. 21D). Therefore, for *A. nidulans* IAT the atypical PTS1 (-ANI) was identified and was shown to be necessary and sufficient for peroxisomal localisation of the protein.

3.2.3. Localisation of IAT in peroxin-mutant strains

Peroxins are proteins required for peroxisome division, for biogenesis from the endoplasmic reticulum, and for the import of proteins into the peroxisomal matrix. The recognition of PTS-containing proteins for subsequent import occurs in the cytosol by specific receptor peroxins (PexE and PexG in *A. nidulans*). They bind the cargo, direct it to the peroxisomal membrane, are translocated to the luminal site of this membrane, release the cargo, and return to the cytosol (reviewed in Platta & Erdmann, 2007).

Recently, different *A. nidulans* peroxin-mutant strains have been characterised (Hynes et al., 2008). Strain *pexC::bar* (Table 2) was unable to form peroxisomal structures at all, which was caused by a *pexC* gene disruption, the yeast *PEX3* orthologue that is essential for initiation of this process (Hoepfner et al., 2005). Due to a deletion / point mutation of the receptor peroxin genes *pexE* and *pexG*, respectively (which are orthologues of the yeast genes *PEX5* [Stanley & Wilmanns, 2006] and *PEX7* [Lazarow, 2006]), other *A. nidulans* mutants (strains *pexE*Δ and *pexG14*; Table 2) were impaired in import of matrix proteins carrying either a PTS1 or a PTS2 sequence. Latter proteins are characterised by a conserved nonapeptide near the N-terminus of the amino acid sequence (Petriv et al., 2004). Recognition of PTS1 proteins by PexE is not affected by the PTS2 receptor PexG and *vice versa*.

To further characterise peroxisomal import of the IAT in *A. nidulans*, localisation of the eGFP-IAT protein fusion was analysed in these peroxin-mutant strains. Therefore, strains *pexC::bar*, *pexE*Δ and *pexG14* were co-transformed using plasmid pALX-aatA and a plasmid

that complemented the respective selectable marker. For *pexC::bar*, plasmid SM6363 carrying an *A. fumigatus* pyridoxine biosynthesis gene that complemented the *pyroA4* mutation (Table 3) was used. In strains *pexE*Δ and *pexG14*, plasmid pabaAnid complemented the Paba$^-$ phenotype. A certain percentage of the prototrophic transformants also carried the *egfp-aatA* gene fusion in different copy numbers (data not shown). One of each was chosen and designated (A) *pexC::bar* GFP-IAT, (B) *pexE*Δ GFP-IAT and (C) *pexG14* GFP-IAT, respectively (Table 2). In brief, they were impaired in (A) peroxisome biogenesis, (B) PTS1 protein import and (C) PTS2 protein import. In Fig. 22, cellular localisation of eGFP-IAT in these mutant strains is shown, as analysed by fluorescence microscopy.

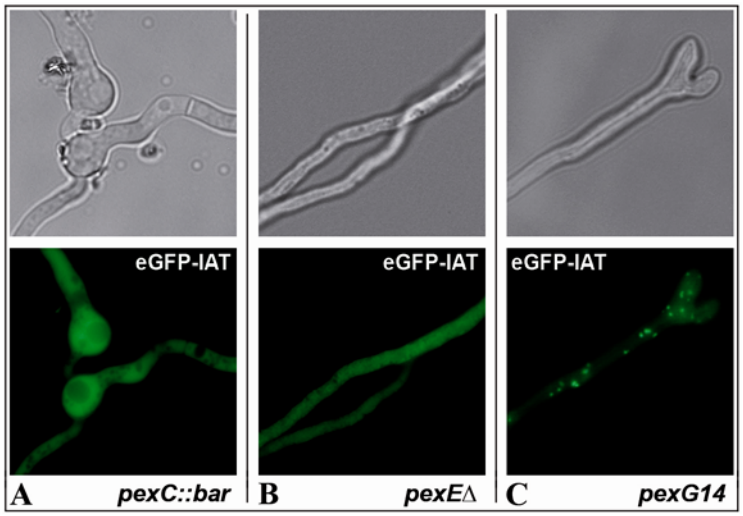

Fig. 22. Cellular localisation of *A. nidulans* IAT in different peroxin-mutant strains. Localisation of eGFP-IAT in genetic backgrounds as followed. (**A**) *pexC::bar*; no peroxisome formation. (**B**) *pexE*Δ; no PTS1-dependent import. (**C**) *pexG14*; no PTS2-dependent import. Light (upper panels) and fluorescence (lower panels) microscopic analyses.

As expected for a peroxisomal protein, mislocalisation of the eGFP-IAT protein fusion was observed in a strain without peroxisomal structures (Fig. 22A). At the same time, this was evidence that the localisation within punctate dots in the wild-type strain (Fig. 21C) reflected localisation in peroxisomes. The eGFP-IAT protein was also mislocated in a strain without PTS1-dependent import (Fig. 22B), whereas localisation was unaffected in strain *pexG14* that lacked PTS2-dependent import (Fig. 22C). Thus, as already expected from the results depicted in Fig. 21, PTS1 and not PTS2 transport was responsible for localising eGFP-IAT to peroxisomes. Taken together, this data showed that the *A. nidulans* IAT is transported to the peroxisomes *via* its atypical PTS1 using the PexE-dependent import machinery.

3.3. Penicillin production of *A. nidulans* strains impaired in peroxisomal IAT localisation

In *P. chrysogenum*, mislocalisation of the IAT has a severe effect on penicillin biosynthesis. Müller et al. (1992) showed that mutants possessing an IAT without the PTS1 no longer produce penicillin although the mutant IAT is active *in vitro*. The authors speculated about a crucial role for peroxisomes in this pathway. To investigate this role in *A. nidulans*, penicillin production was assayed in both peroxin mutants and in a strain with an *aatA* allele lacking the PTS1-encoding sequence.

3.3.1. Analysis of peroxin mutant strains

As indicated above, the involvement of peroxisomes in penicillin biosynthesis has been primarily investigated in *P. chrysogenum*. But despite some efforts, peroxin mutants equivalent to the *A. nidulans* ones could not be generated in this fungus – and therefore, research stagnated at this point. The approach to disrupt the *pexE* orthologue *Pc-pex5* yielded genetically unstable mutants that were impaired in growth (Kiel et al., 2004). Other mutants, e.g., of *pexA* and *pexF* orthologues encoding ATPases that are important for PTS1 and PTS2 protein import (Hynes et al., 2008), could not be isolated at all (Kiel et al., 2000). However, overproduction of Pc-Pex11p, a peroxin that is involved in peroxisome proliferation, resulted in a massive rise in peroxisome quantity and a twofold increase in penicillin production by *P. chrysogenum* (Kiel et al., 2005), again demonstrating the importance of these organelles.

Since in *A. nidulans* the respective mutants were viable (Hynes et al., 2008), their penicillin producing potential could be analysed. In Table 6, these strains are listed. Proposed function of the affected protein, peroxisome status and IAT localisation within the mutants, either presumed or verified (Fig. 22), are also indicated. In brief, *pexC::bar* is impaired in peroxisome formation, while *pexK*Δ (deletion of the *Pc-Pex11* orthologue) possesses a few of the organelles but does not respond to fatty acid induced proliferation (Hynes et al., 2008). All other mutants lack proper import of matrix proteins, either of all PTS1- and PTS2-containing proteins, or of only one of the two types.

Table 6. *A. nidulans* peroxin-mutant strains analysed for penicillin production. Except for IAT, all data according to Hynes et al. (2008; information labelled by * was not directly specified in the text of the publication, but deduced from the figures therein).

strain	impaired function	peroxisome appearance	IAT localisation
pexC::bar	peroxisome biogenesis	no peroxisomes	cytosolic
*pexK*Δ	peroxisome proliferation	only few larger peroxisomes	likely peroxisomal
pexA9	PTS1+PTS2 protein import	not analysed	likely cytosolic

pexF23	PTS1+PTS2 protein import	"membrane ghosts"	likely cytosolic
pexM15	PTS1+PTS2 protein import	not analysed	likely cytosolic
*pexE*Δ	PTS1 recognition / import	normal appearance*	cytosolic
pexG14	PTS2 recognition / import	normal appearance*	peroxisomal

Penicillin production of all strains was assayed and compared to the respective wild-type strains, whose production was afterwards set to 100% to enable comparison of the different mutant strains. Results are summarised in Fig. 23.

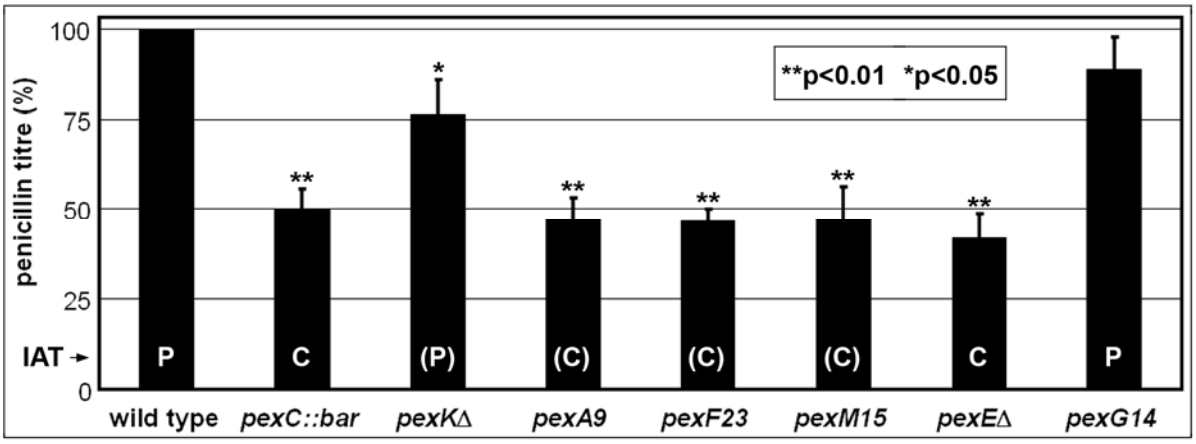

Fig. 23. Penicillin production of *A. nidulans* peroxin-mutant strains. Calculated penicillin titres with the respective wild-type strains set 100%. Statistic significance is indicated by the p-value. Characteristics of the mutants are listed in Table 6, (presumed) IAT localisation is indicated (P, peroxisome; C, cytosol).

Whenever localisation of the IAT was affected, i.e., the enzyme was located in the cytosol (see also Table 6), the penicillin titre was decreased. Thus, as previously observed for *P. chrysogenum*, peroxisomal localisation appears to be beneficial. But surprisingly, a strain without peroxisomes (*pexC::bar*) was still able to produce penicillin, which was also observed for the mutant possessing only "membrane ghosts" instead of peroxisomes (*pexF23*). Similarly, the penicillin titre of strains lacking functional PTS1 protein import (i.e., *pexA9*, *pexF23*, *pexM15*, and *pexE*Δ) was reduced to 50% compared to wild-type levels, whereas mutation of only the PTS2 receptor in strain *pexG14* had no significant effects. This suggested that, in *A. nidulans*, transport of the peroxisomal proteins involved in penicillin biosynthesis, directly or indirectly, is mainly PTS1- and not PTS2-dependent. But apparently, proper localisation of these proteins and, moreover, the presence of functional peroxisomes at all is not absolutely required for penicillin production in *A. nidulans*. For IAT as a PTS1-containing enzyme, this had to be confirmed in more detail (see below).

Interestingly, a strain with reduced number of peroxisomes due to lacking division and proliferation (*pexK*Δ) showed an intermediate reduction and produced about 75% of the wild-

type level. This indicated that the peroxisomes of this mutant are functional in terms of enabling elevated penicillin production compared to strains without peroxisomes. However, either the lower total number or other unknown peroxisome-related impairments of this mutant apparently prevented wild-type production levels.

3.3.2. Generation and analysis of an IAT$^{\Delta PTS}$ mutant strain

The previous experiments showed that efficient PTS1 protein import and functional peroxisomes were not absolutely required for penicillin biosynthesis in *A. nidulans*. The IAT of this fungus thus appeared to be also functional in the cytoplasm, which would be in contrast to the data obtained for *P. chrysogenum*. To investigate this further, the *aatA* gene of *A. nidulans* wild-type strain TN02A21 (Table 2) was replaced by an *aatA* allele lacking the PTS1-encoding sequence. Therefore, plasmid pbar-aatA$^{\Delta PTS1}$ was generated by cutting a 5'-truncated *aatA*$^{\Delta PTS1}$ fragment from plasmid pALX- aatA$^{\Delta PTS1}$ and ligating this fragment into the *Eco*RI-linearised vector SM6355 (Table 3). This plasmid also carried the *bar* gene as a selectable marker to confer glufosinate resistance (Nayak et al., 2006). Since non-homologous integration of DNA was reduced in strain TN02A21 (Δ*nkuA*; Nayak et al., 2006), upon transformation with plasmid pbar-aatA$^{\Delta PTS1}$ the truncated *aatA*$^{\Delta PTS1}$ construct could be directly targeted to the endogenous *aatA* gene. By integration of the whole plasmid into the genome after a single-crossover event, the *aatA* locus was disrupted, yielding an intact copy of the gene with the deleted PTS1-encoding sequence (*aatA*$^{\Delta PTS1}$) and a non-functional 5'-truncated copy, both separated by the *bar* gene. This was confirmed by Southern blot analysis (Fig. 24A), and one of the thus modified transformants was designated AatA$^{\Delta PTS1}$ (Table 2).

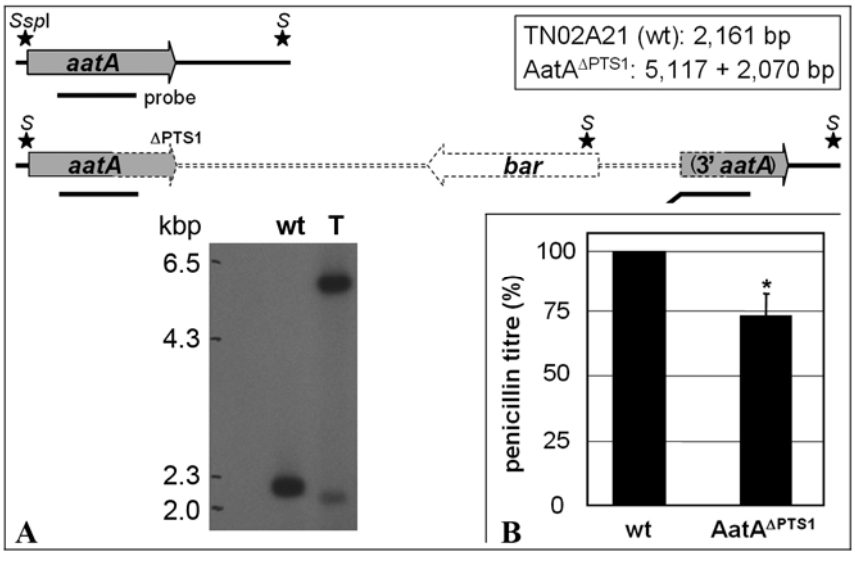

Fig. 24. Generation and analysis of *A. nidulans* strain AatA$^{\Delta PTS1}$. (**A**) Schematic maps of the chromosomal *aatA* loci (upper part) and respective Southern blot analysis (lower part). The integration of plasmid pbar-

AatA$^{\Delta PTS1}$ (dashed lines) yielded both the modified *aatA*$^{\Delta PTS1}$ gene and a promoterless 3'-*aatA* by-product. After *Ssp*I (★) restriction, hybridisation to the 673 bp probe (obtained by PCR using oligonucleotides aatA-disrupt-5' and aatA-disrupt-3'; Table 4) resulted in the indicated signal sizes for the wild-type strain TN02A21 (wt) and the transformed strain AatA$^{\Delta PTS1}$ (T). (**B**) Penicillin production. Wild-type strain (see text for details) set 100%.

According to the localisation studies of strain GFP-IAT$^{\Delta PTS1}$ (Fig. 21D), the IAT of strain AatA$^{\Delta PTS1}$ was mislocated to the cytosol. Penicillin production of this strain was assayed and, to exclude effects of the disruption cassette or the glufosinate resistance, was compared to a strain that was generated in line with AatA$^{\Delta PTS1}$, but starting from pALX-aatA. Thus, this strain possessed a peroxisomal IAT in the same genetic background as strain AatA$^{\Delta PTS1}$. As expected, penicillin production of this strain was not different from that of the wild-type strain TN02A21 (data not shown). Therefore, it was considered as wild type in Fig. 24B. Compared to that strain, penicillin production of strain AatA$^{\Delta PTS1}$ was significantly ($p < 0.05$) reduced to 75%, confirming the conclusion drawn from the peroxin-mutant strains, that the *A. nidulans* IAT is also functional in the cytoplasm. The observed difference to those peroxin-mutant strains that also possessed a mislocated IAT (and produced about 50% of the wild-type level; Fig. 23) was likely caused by the mislocalisation of additional, otherwise peroxisomal proteins in these strains, which provoked metabolic impairments that might indirectly influenced penicillin production. In this context, putative acyl-CoA ligases as side-chain activators could play a role (see discussion). By contrast, in strain AatA$^{\Delta PTS1}$ only the IAT was mislocalised and thus separated from the beneficial but obviously not essential peroxisomal environment. Assuming that in *A. nidulans* the compartmentalisation of the biosynthesis pathway is similar to that in *P. chrysogenum* (Fig. 19), i.e., with part of the (activated) substrates in the peroxisomes, it was rather surprising to detect only a relatively moderate decrease in penicillin production in strain AatA$^{\Delta PTS1}$. It is apparent that in *A. nidulans* the last step of penicillin biosynthesis is not absolutely dependent on peroxisomal localisation of IAT, and also not that restricted to these organelles, as it is in the industrial producer *P. chrysogenum* – an unexpected difference that requires further investigation.

Taken together, despite its unusual PTS1, the *A. nidulans* IAT is located within peroxisomes. In contrast to *P. chrysogenum*, this localisation is not necessary for penicillin production *per se*, which is also true for the presence of functional peroxisomes. However, in *A. nidulans* compartmentalisation of the final step of this pathway is nevertheless advantageous with respect to the final penicillin yield.

4. *aatB* and evolution of *aatA*

As outlined in the introduction and the previous chapters, the pathway-specific *aatA* gene has an outstanding position within penicillin biosynthesis. Interkingdom horizontal gene transfer (HGT) from bacteria is the predominant hypothesis on the evolutionary origin of the first two genes of the pathway, i.e., *acvA* and *ipnA*. For the *aatA* gene a eukaryotic origin and thus, recruitment of the gene to the *acvA-ipnA* gene cluster during evolution appears to be more likely. However, the ancestry of the *aatA* gene is still not elucidated.

4.1. Generation and analysis of an *A. nidulans aatA*-disruption strain

Until now, the characterisation of the *A. nidulans aatA* gene was rather restricted to the analysis of its regulation (e.g., Litzka et al., 1995; Caruso et al., 2002), and did hardly involve the importance of the gene itself for penicillin biosynthesis. To demonstrate the need of an intact *aatA* gene for penicillin production, the gene was disrupted in the *A. nidulans* wild-type strain TN02A21 (Fig. 25).

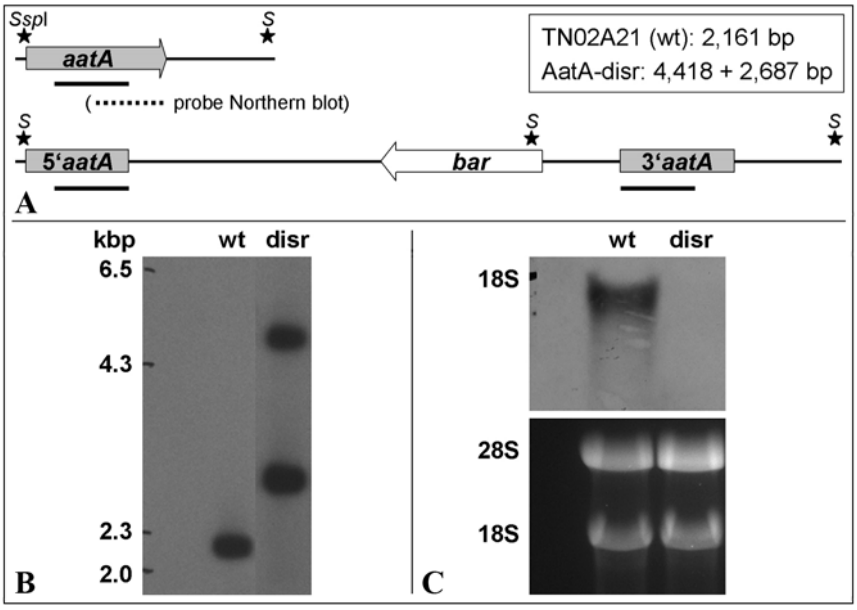

Fig. 25. Generation of an *A. nidulans aatA*-disruption strain. (**A**) Schematic map of the chromosomal *aatA* locus of the wild-type strain (TN02A21) and the *aatA*-disruption mutant (AatA-disr). *Ssp*I cleavage sites (★) and expected signal sizes are indicated. Positions to which the probes hybridise are marked by a continuous (Southern blot analysis) and a dashed line (Northern blot analysis). (**B**) Southern blot analysis. Chromosomal DNA was cut by *Ssp*I and hybridised to the probe indicated in (A). Bands characteristic of the altered locus were detected for AatA-disr (disr). (**C**) Northern blot analysis of *aatA* transcripts. RNA was hybridised to the probe indicated in (A). Position of the 18S rRNA on the membrane is indicated. rRNA bands are shown as loading control.

Plasmid pbar-aatA-disrupt (Table 3) was generated by amplifying a 673 bp central part of the *aatA* gene using oligonucleotides aatA-disrupt_5' and aatA-disrupt_3' (Table 4), and subsequent ligation of the DNA fragment into the *Eco*RV linearised vector SM6355 carrying the glufosinate resistance gene *bar* as selectable marker (Table 3). The amplified DNA fragment was later on also used as probe for Southern blot analysis (indicated in Fig. 25A). Due to the *nkuA* mutation of strain TN02A21 (Table 2), upon transformation, plasmid pbar-aatA-disrupt was directly targeted to the *aatA* locus. By a single-crossover event the gene was disrupted, leaving both a truncated 5' and 3' *aatA* fragment (Fig. 25A). All glufosinate-resistant strains that were subjected to Southern blot analysis carried this *aatA* disruption, and one of them (Fig. 25B) was chosen and designated AatA-disr.

To verify the effect of the *aatA* disruption on *aatA* gene expression, Northern blot analysis of both the wild type and the disruption strain was performed (Fig. 25C). RNA was isolated after incubation of the strains for 48 h in AMM. It was hybridised to the 711 bp probe indicated in Fig. 25A (dashed line), which was generated by amplification of the fourth exon of the *aatA* gene using oligonucleotides aatA_exon4_up and aatA_exon4_down (Table 4). Transcripts of the *aatA* gene could only be detected in the wild-type strain TN02A21, whereas the missing signal of strain AatA-disr was indicative of lacking transcription (Fig. 25C).

Penicillin titres of both strains and the complemented strain A-disr_aatA in which the *aatA* gene under the control of the native promoter was re-introduced (Table 2), were measured by bioassay (Fig. 26A).

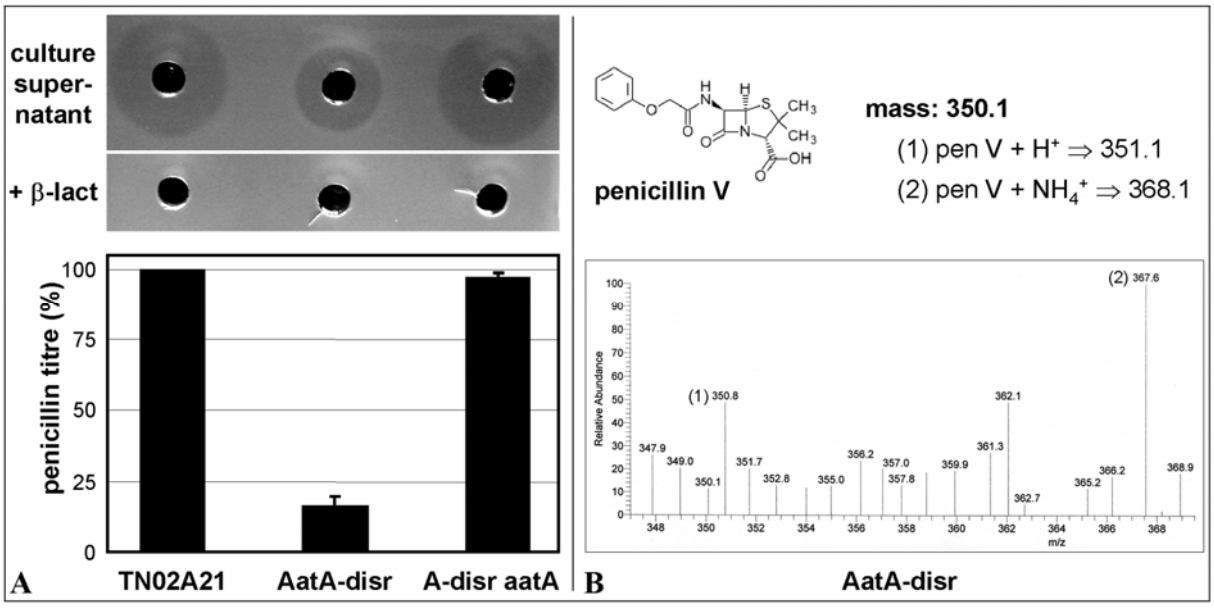

Fig. 26. Penicillin production of an *aatA*-disruption strain. (**A**) Penicillin titres of *A. nidulans* strains TN02A21, AatA-disr and the complemented strain A-disr_aatA. Upper panels show growth inhibition of *Bacillus calidolactis* caused by the 1:10 diluted supernatant of the strains incubated in FM for 72 h, before and

after treatment with β-lactamase (β-lact). The diagram shows the calculated penicillin titres with the wild-type strain TN02A21 set 100 %. The reduced penicillin titre of strain AatA-disr was restored in the complemented strain A-disr_aatA. (**B**) LC-MS analysis of culture supernatant extracts of strain AatA-disr. The indicated penicillin V adducts derived from protonated (1) and ammonium-carrying (2) derivatives, respectively, were detectable after a retention time of 33.0 ± 0.5 min.

For complementation of the disruption, the *aatA* gene including its native promoter was amplified using oligonucleotides aatAp_up and aatA_down (Table 4). The 2,037 bp DNA fragment was ligated into plasmid SM6363 to give plasmid pAfpyro-aatA_natp (Table 3). Since the vector-backbone of this plasmid contained large homologous sequences to pbar-aatA-disrupt (pBS-SK$^+$ derivatives), the *aatA* gene was re-introduced at the *aatA*-disruption locus of strain AatA-disr to yield strain A-disr_aatA, which was confirmed by Southern blot analysis (data not shown).

As expected, in a bioassay growth inhibition of the sensitive *Bacillus calidolactis* strain was reduced in the *aatA*-disruption mutant compared to the wild-type strain (Fig. 26A). This effect was not visible in the complemented strain. β-Lactamase treatment identified the inhibiting substance as a β-lactam. First, it was assumed to consist of isopenicillin N (IPN), the expected product of ACVS and IPNS activity (see Fig. 2). But surprisingly, further LC-MS analysis of the culture supernatant of strain AatA-disr showed that the *aatA*-disruption strain was still able to produce penicillin V (Fig. 26B).

4.2. Characterisation of the *aatB* gene, a putative paralogue of *aatA*

The fact that an *A. nidulans aatA* mutant was still able to convert IPN to penicillin V indicated the existence of a second gene which, at least in part, complements the *aatA* disruption. Therefore, using the *aatA* DNA sequence as template the *A. nidulans* genome was analysed by BLAST search (*blastn*). AN6775.3 located on chromosome I and thus outside of the penicillin biosynthesis gene cluster, which is located on chromosome VI (see Fig. 3), was found as the only hit (Fig. 27A).

4.2.1. Structural comparison of *aatA* and AN6775.3 (*aatB*)

AN6775.3 possessed 58 % similarity at the nucleotide level with the *aatA* gene and also a similar length of 1,196 bp, compared to 1,241 bp of *aatA*, with nearly identical sizes and distribution of exons (Fig. 27B). Due to these characteristics the gene was designated *aatB*. *In silico* analysis of the adjacent genes gave no hint for the presence of a known or putative secondary metabolite cluster. Sequence comparison of the encoded AatB protein, comprising 345 amino acid residues, and IAT encoded by the *aatA* gene revealed 41.7 % amino acid

identity and 55.2 % similarity, respectively (Fig. 27C). In addition, similar to IAT AatB possessed both an acyl-CoA : 6-aminopenicillanic acid acyltransferase (AAT) domain spanning amino acid residues 117 to 333, and the putative DGC cleavage motif, but lacked the peroxisomal targeting signal 1 (PTS1; see results chapter 3.1.) suggesting a cytoplasmic localisation of AatB. Taken all the similarity data together, *aatA* and *aatB* are most likely paralogous genes.

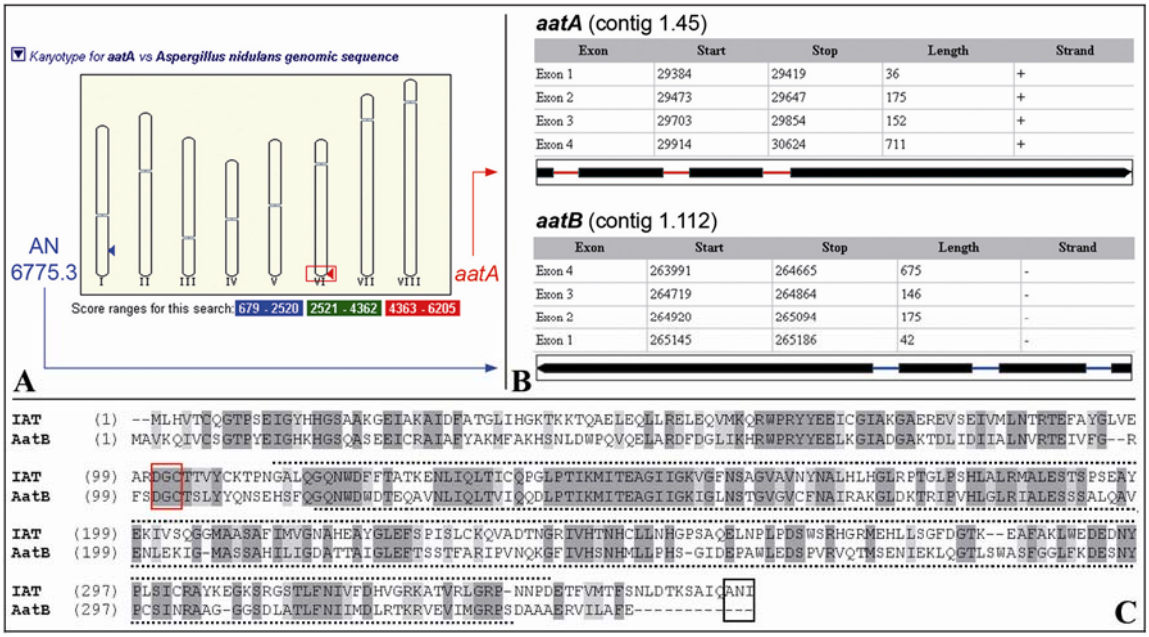

Fig. 27. Structural comparison of *aatA* and *aatB*, and of the encoded proteins IAT and AatB. (**A**) Chromosomal localisation of the genes obtained by *blastn* search using the *A. nidulans aatA* gene as query and the *A. nidulans* genomic sequence as template. A single hit yielded AN6775.3 (designated *aatB*), i.e., there are no other putative paralogues. (**B**) Comparison of the (for *aatB* predicted) exon sizes and distribution within *aatA* and *aatB*. Exons are illustrated by black boxes. (**C**) Sequence alignment of the encoded proteins IAT and AatB. Identical amino acids are shaded in dark grey, similar amino acids in light grey. The AAT domains (see text) are marked by dashed lines. The putative DGC cleavage motif (red) and the PTS1 of IAT (black) are boxed.

4.2.2. Phylogenetic analysis of *aatA* and *aatB*

Since *aatA* and *aatB* are putative paralogous genes in *A. nidulans* that, *per definitionem* (Fitch, 2000), derived from duplication of a common ancestral gene, it was of interest to study the genes' phylogeny, i.e., to look for homologues in other fungi.

For the *aatA* gene, only one homologue has been characterised so far, namely the *penDE* gene of *P. chrysogenum* (Barredo et al., 1989; Tobin et al., 1990). Thus, genomes of other fungi were searched for the occurrence of homologues of both genes, *aatA* and *aatB*. Fig. 28 shows a phylogenetic analysis of the encoded homologous proteins, as they were found within the fungal kingdom. For all proteins, an AAT domain was predicted.

Homologues not possessing a PTS1 and whose encoding gene was not part of a cluster were widely distributed mainly within the Euascomycota. Hence, despite not further characterised yet, they structurally shared more characteristics with *aatB* /AatB than with *aatA* /IAT. Thus, they could be considered as AatB-like. Their phylogeny reflected ordinary evolution, with the *A. nidulans* AatB homologue at some distance to the other Aspergilli (Galagan et al., 2005).

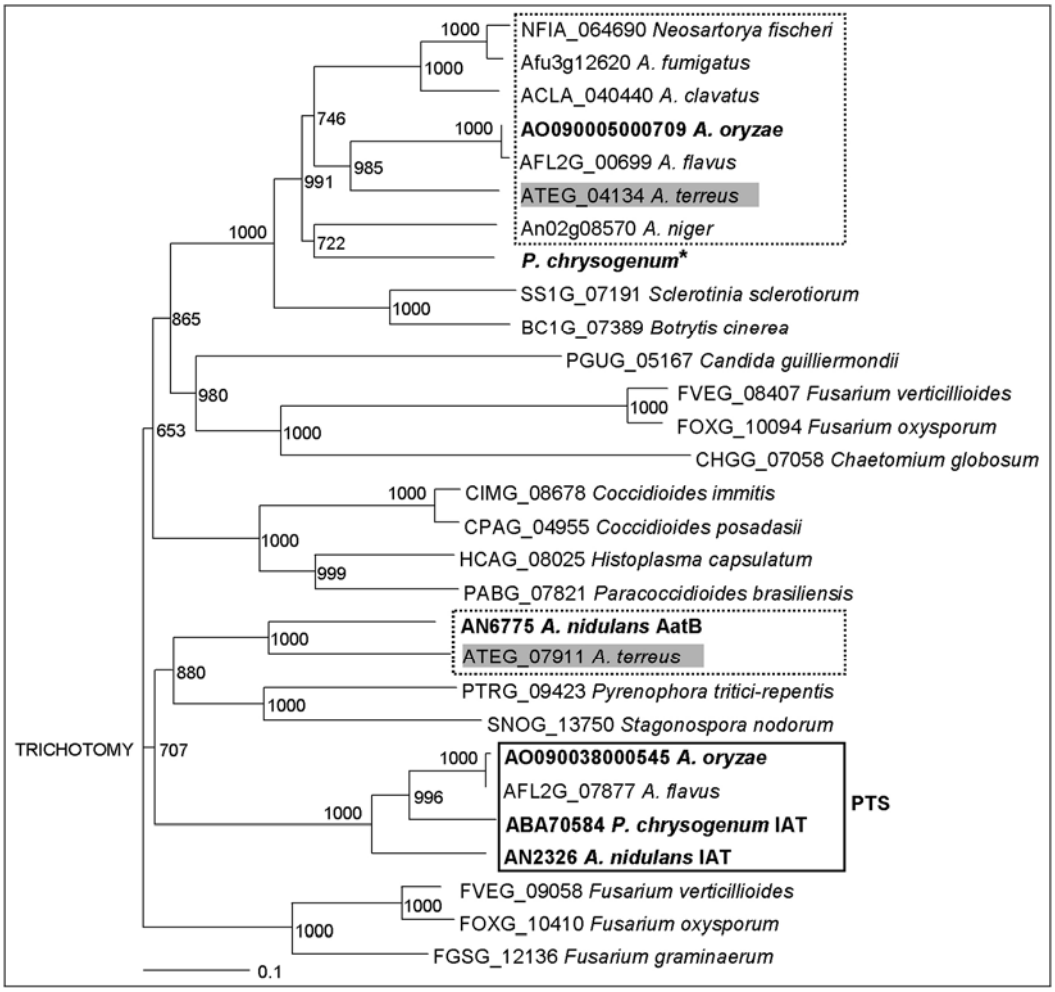

Fig. 28. Phylogenetic distribution of AatB and IAT homologues within fungi. Amino acid sequences were obtained from Broad Institute using *blastp*. Locus IDs are listed on the right side of the tree (**P. chrysogenum*: AatB-like, personal communication M. van den Berg). The IAT proteins possessing a PTS1 form a separate clade (boxed) and are only found in fungi that also have an AatB or AatB-like protein. All other listed proteins are presumably AatB-like (see text). Phylogeny of AatB and AatB-like proteins within the Aspergilli is marked by dashed boxes. *A. terreus* has two putative AatB-like proteins (shaded), one of them grouped with the *A. nidulans* AatB close to the IAT proteins. Sequences shown in bold are further analysed in Fig. 29.

Interestingly, the homologues having a PTS1 (and forming a separate clade) were only found in fungi that already had an AatB(-like) copy and, moreover, that also possessed the penicillin biosynthesis gene cluster (also summarised in Table 7; see results chapter 4.6.4.). In these fungi, the genes encoding the PTS1-containing proteins were exclusively found as part

of this gene cluster associated with the respective *acvA* and *ipnA* homologues. Thus, they could be considered as *aatA* homologues, and the encoded proteins as homologues of IAT. As specified in Fig. 29A1, the *aatA* homologues of *A. nidulans*, *A. oryzae* (AO090038000545) and *P. chrysogenum* (*penDE*) showed identical exon distribution and similar exon sizes. The encoded proteins all possessed a PTS1 (Fig. 29C), with the *A. oryzae* and *P. chrysogenum* sequences being more consistent with the consensus S/A R/K L/M than the *A. nidulans* one.

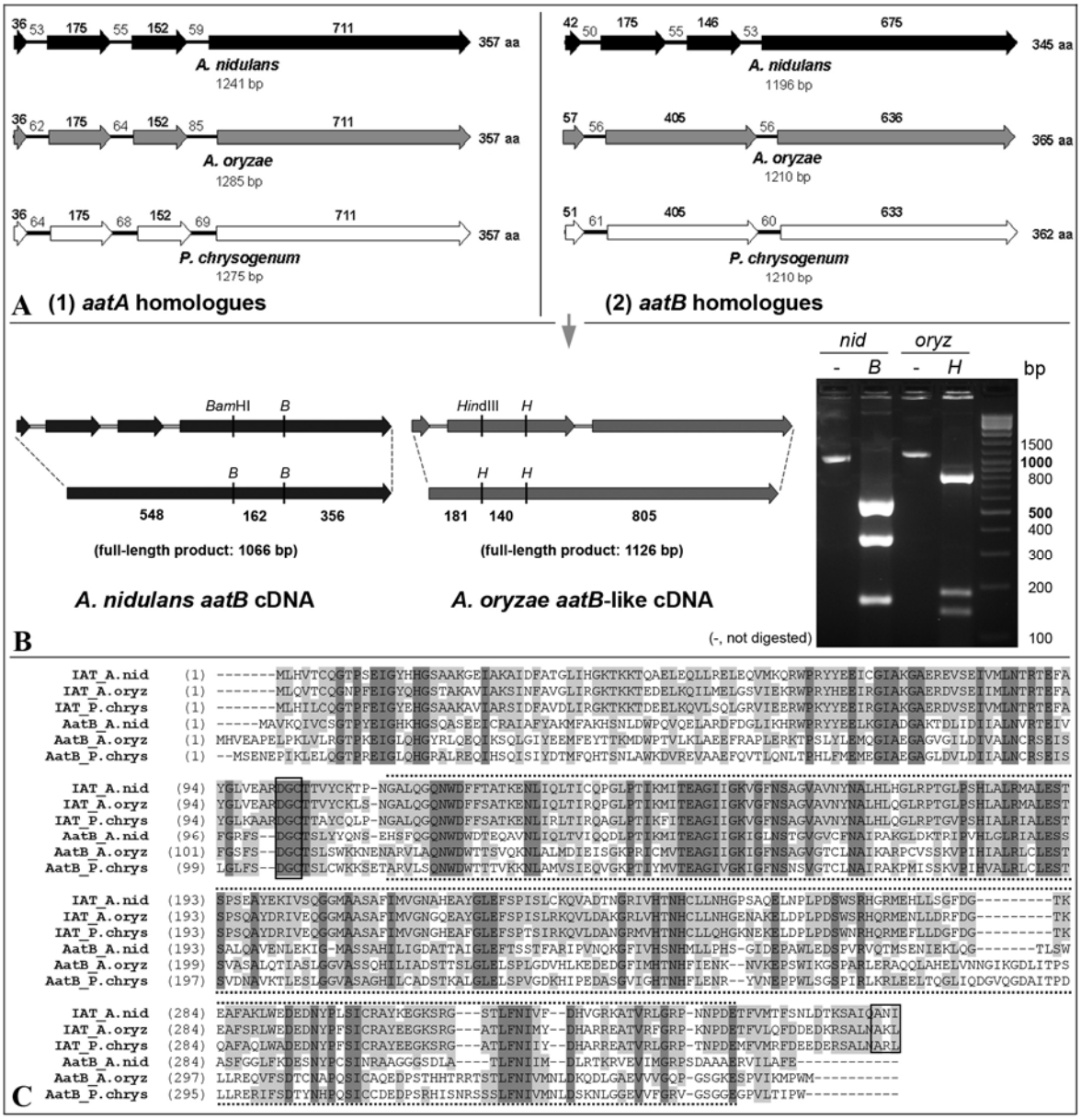

Fig. 29. Structural comparison of *aatA* and *aatB* homologues of *A. nidulans*, *A. oryzae* and *P. chrysogenum*. For locus ID and phylogenetic position see Fig. 28. (**A**) Exon distribution within the *aatA* (1) and *aatB* (2) homologues. Gene sizes and sizes of exons (arrows) and introns (lines) are indicated. The numbers at the right side show the length of the encoded amino acid sequence depicted in (C). (**B**) Confirmation of the predicted exon distribution of the Aspergilli *aatB* homologues (shown in A2) by RT-PCR and restriction digest. cDNA was amplified from mRNA of *A. nidulans* TN02A21 and *A. oryzae* DSM 1862 (Table 2) using primer pairs AnidaatB_up / AnidaatB_down and AoryzaatB_up / AoryzaatB_down, respectively (Table 4). Products of the

indicated sizes were subjected to *Bam*HI (*B*) and *Hin*dIII (*H*) digest, respectively. Separation of the DNA fragments on a 2 % (w/v) agarose gel yielded the sizes expected for the predicted exon distributions. (**C**) Sequence alignment of the IAT and AatB(-like) homologues. Identical amino acids are shaded in dark grey, similar amino acids in light grey. The (putative) AAT domains are marked by dashed lines. The (putative) DGC cleavage site is also conserved, whereas only the IATs possess a PTS1-containing C-terminus (both boxed).

The *aatB* gene of *A. nidulans* and the putative *aatB* homologues of *A. oryzae* (AO090005000709) and *P. chrysogenum* (Marco van den Berg, personal communication) were analysed in more detail in Fig. 29A2. As their counterparts from other fungi, which do not possess the cluster, they were located outside of the penicillin biosynthesis gene cluster, and in contrast to the IAT homologues, none of the predicted AatB proteins possessed a PTS1 (Fig. 29C). The exon distribution of the *A. nidulans aatB* was very similar to all *aatA* genes, whereas the putative *aatB* homologues of *A. oryzae* and *P. chrysogenum* shared a different pattern. This finding was confirmed for the *aatB* genes of both Aspergilli by RT-PCR, control restriction digest of the PCR product (Fig. 29B) and subsequent sequencing. This intermediate position of *A. nidulans aatB* was also reflected by its phylogenetic position (Fig. 28) and by the encoded proteins: all IAT proteins showed a very high sequence identity (about 80 %), whereas the AatB homologues of *A. oryzae* and *P. chrysogenum* possessed more than 60 % identity. AatB of *A. nidulans* showed sequence identities of about 40 % with both the other AatB proteins and all IAT homologues. Furthermore, the different evolutionary path of the *A. nidulans aatB* gene was also illustrated by the fact that it does show synteny with the other *aatB* genes of the Aspergilli (data not shown).

Since both proteins, IAT and AatB, showed significant sequence and structural identities, it is conceivable that *aatA* and *aatB* are descendants of a common ancestral gene with *aatA* being only present as part of the cluster in the penicillin producers. Therefore, the *aatA* gene most likely derived from a duplication and specification event yielding a gene that was, later on or in between, recruited to the *acvA-ipnA* gene cluster. Interestingly, some other fungi, e.g., *A. terreus* and *Fusarium* species, also possessed an additional copy of an *aatB*-like gene (Fig. 28), but in those cases the genes neither encoded a PTS1 nor were part of a gene cluster, obviously having a different evolutionary history than *aatA*. According to the phylogenetic tree in Fig. 28 this additional *A. terreus* protein exhibited more similarity to the *A. nidulans* AatB and to the IAT homologues than the other copy.

4.2.3. Generation and analysis of an *A. nidulans aatB*-disruption strain

It was likely that the putative *aatA* paralogue *aatB* was responsible for the residual penicillin production observed for the *aatA*-disruption strain (Fig. 26). To study the influence

of *aatB* on the penicillin titre of *A. nidulans*, the *aatB* gene was disrupted in the *A. nidulans* wild-type strain TN02A21 (Fig. 30). For this purpose, plasmid pAfribo-aatB-disrupt (Table 3) was generated by amplifying a 660 bp central part of the *aatB* gene using primers aatB-disrupt_5' and aatB-disrupt_3' (Table 4). The amplified DNA fragment was ligated into plasmid SM6392 carrying the *A. fumigatus* riboflavin biosynthesis gene as selectable marker (Table 3), and was later on also used as probe for Southern blot analysis (indicated in Fig. 30A). Upon transformation of the *nkuA* mutant strain TN02A21 (Table 2), plasmid pAfribo-aatB-disrupt was directly targeted to the *aatB* locus. By a single-crossover event the gene was disrupted, leaving both a truncated 5' and 3' *aatA* fragment (Fig. 30A). All riboflavin-prototrophic strains that were subjected to Southern blot analysis carried this *aatB* disruption, and one of them (Fig. 30B) was chosen and designated AatB-disr (Table 2).

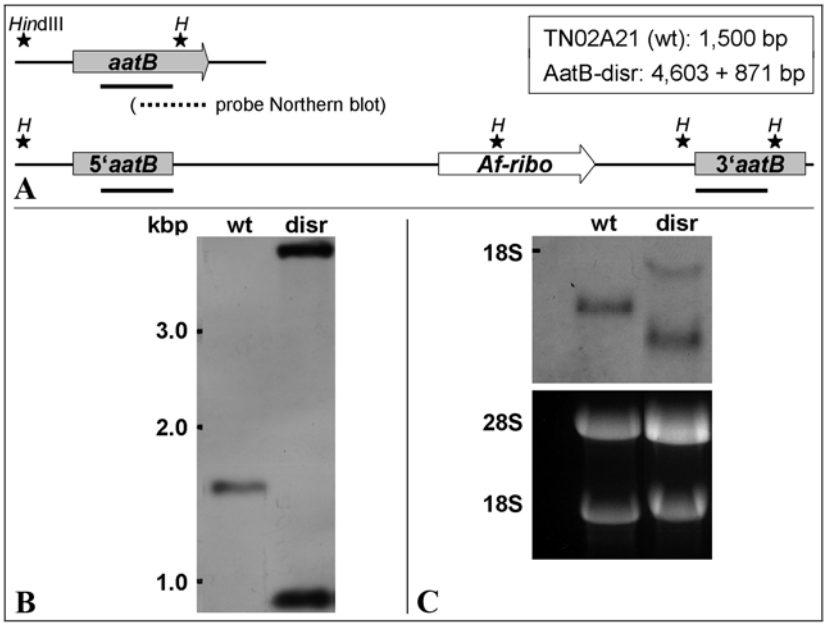

Fig. 30. Generation of an *A. nidulans aatB*-disruption strain. (**A**) Schematic map of the chromosomal *aatB* locus of the wild-type strain (TN02A21) and the *aatB*-disruption mutant (AatB-disr). *Hin*dIII cleavage sites (★) and expected signal sizes are indicated. Positions to which the probes hybridise are marked by a continuous (Southern blot analysis) and a dashed line (Northern blot analysis), respectively. (**B**) Southern blot analysis. Chromosomal DNA was cut by *Hin*dIII and hybridised to the probe indicated in (A). Bands characteristic of the altered locus were detected for AatB-disr (disr). (**C**) Northern blot analysis of *aatB* transcripts. RNA was hybridised to the probe indicated in (A). Position of the 18S rRNA on the membrane is indicated. rRNA bands are shown as loading control.

To monitor expression of the *aatB* gene and thus, verify its disruption, strains TN02A21 and AatB-disr were subjected to Northern blot analysis (Fig. 30C). RNA of the strains was isolated after 48 h incubation in AMM and hybridised to the 672 bp probe corresponding to the fourth exon of the gene (indicated in Fig. 30A), that was amplified using oligonucleotides

aatB_exon4_up and aatB_exon4_down (Table 4). Transcripts of the complete *aatB* gene could only be detected for the wild type. For the *aatB*-disruption strain, the truncated transcript most likely derived from the incomplete 5' region of the *aatB* gene and the longer transcript most probably resulting from a read-through transcript of the further upstream located *A. fumigatus* riboflavin biosynthesis gene were conceivably non-functional. The *aatB* transcript was shorter than that of the *aatA* gene (see Fig. 25C).

Penicillin titres of the wild type, the *aatB*-disruption mutant and the complemented strain B-disr_aatB in which the *aatB* gene under the control of the native promoter was re-introduced, were measured by bioassay (Fig. 31A, upper panel). For complementation of the disruption, the *aatB* gene was amplified using oligonucleotides aatBp_up and aatB_down (Table 4). The 2,392 bp DNA fragment was ligated into plasmid SM6363 to give pAfpyro-aatB_natp (Table 3). Since the vector-backbone contained large homologous sequences to pAfribo-aatB-disrupt, the *aatB* gene was re-introduced at the *aatB*-disruption locus of strain AatB-disr, which was confirmed by Southern blot analysis (data not shown).

In the bioassay, the reduction of the penicillin titre was not as clearly visible as with the *aatA*-disruption mutant. However, further calculation revealed that in the *aatB*-disruption strain the penicillin titre, being mainly penicillin V as analysed by LC-MS (Fig. 31A, lower panel), was reduced to approximately 55 %. As this effect could be reversed in the complemented strain B-disr_aatB, it can be concluded that disruption of the *aatB* gene affected the penicillin titre even in the presence of a functional *aatA* gene.

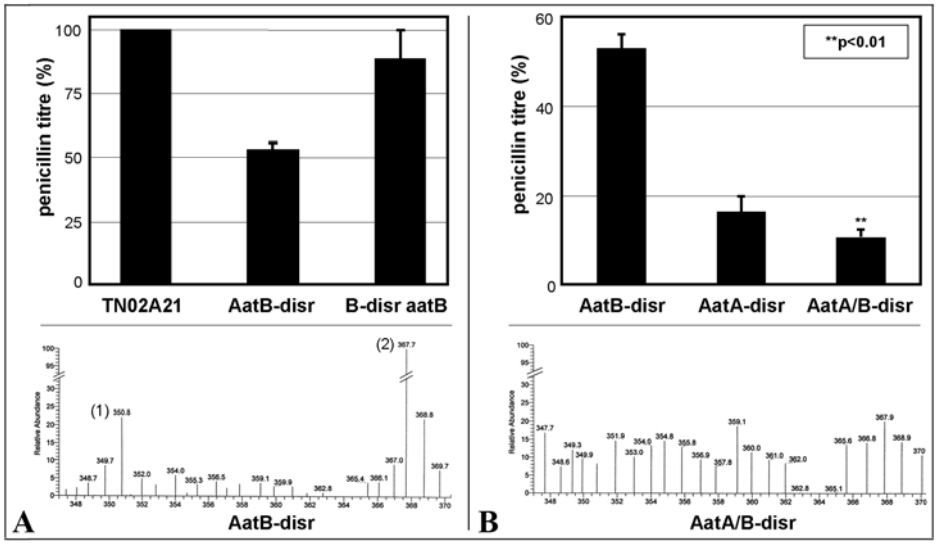

Fig. 31. Penicillin production of *aatB*-disruption strains. (**A**) Upper panel: Penicillin titres of *A. nidulans* strains TN02A21, AatB-disr and the complemented strain B-disr_aatB. Calculated penicillin titres with the wild-type strain TN02A21 set 100 %. The reduced penicillin titre of strain AatB-disr was restored in strain B-disr_aatB. Lower panel: LC-MS analysis of culture supernatant extracts of strain AatB-disr. Penicillin V adducts

(1) and (2) were detectable after a retention time of 33.0 ± 0.5 min. Note the cut scale and the therefore higher abundance of the adducts compared to strain AatA-disr in Fig. 26B. (**B**) Upper panel: Penicillin titres of *A. nidulans* strains AatA-disr, AatB-disr and the double-disruption strain AatA/B-disr. Calculated penicillin titres with the wild-type strain TN02A21 (not shown) set 100 %. Statistical significance of data obtained for AatA/B-disr compared to AatA-disr is indicated by the p-value. Lower panel: LC-MS analysis of culture supernatant extracts of strain AatA/B-disr. No penicillin V adducts were detected.

To investigate the influence of disruption of *aatB* in an *aatA*-disruption strain, strains AatA-disr and AatB-disr were crossed and resulting progeny was screened by Southern blot analysis for strains carrying both disruptions. One of these double-disruption mutants was designated AatA/B-disr (Table 2). As depicted in Fig. 31B (upper panel), the penicillin titre of this mutant compared with the single-disruption mutants was significantly decreased ($p < 0.01$). Moreover, penicillin V was no longer detectable by LC-MS in the double-disruption strain (Fig. 31B, lower panel). Therefore, the residual inhibition observed in bioassays, that was completely β-lactamase sensitive, most likely resulted from IPN that could be formed without the enzymatic activity of IAT or AatB (see Fig. 2). Taken together, disruption of the *aatB* gene affected the penicillin titre and, in combination with an *aatA* mutation further reduced the penicillin titre below detection level. Hence, the encoded proteins of both genes were capable of catalysing the same enzymatic reaction, i.e., the conversion of IPN to penicillin V. Obviously, both enzymes were necessary and sufficient for the overall penicillin production of *A. nidulans*.

4.3. Comparison of IAT and AatB: localisation and processing

As depicted in Fig. 27C, the *A. nidulans* IAT and AatB possess conserved domains like the AAT domain responsible for the enzymatic activity, and the DGC motif for cleavage of the proteins – although for IAT this was suggested to occur only inefficiently (Fernández et al., 2003). However, the main difference consists in the C-terminus that only within the IAT comprises a PTS1 being necessary for peroxisomal localisation of the protein (see results chapter 3.2.). To further characterise AatB, the protein was compared to IAT with respect to cellular localisation and processing.

4.3.1. Cellular localisation of AatB

AatB was expected to be a cytosolic protein since obvious targeting signals were missing in the amino acid sequence. To confirm this, localisation of an N-terminal eGFP fusion protein corresponding to eGFP-IAT (Fig. 21) was analysed. For this purpose, plasmid pALX-aatB encoding an eGFP-AatB fusion under the control of the *gpdA* promoter (Table 3;

Fig. 32A) was generated by ligation of a 1,586 bp *aatB* DNA fragment (amplified using oligonucleotides aatB_up_XmaI and aatB_down_XbaI, Table 4) in frame with *egfp* into the *Xma*I/*Xba*I-linearised vector pALX213. Using plasmid pabaAnid as co-transforming agent, *A. nidulans* strain AXB4A2 was transformed to *p*-aminobenzoic acid prototrophy, thus generating strain AX_GFP-AatB (Table 2). To have a control with the same genetic background, plasmid pALX-aatA encoding the eGFP-IAT fusion was used to generate strain AX_GFP-IAT (Table 2). It was furthermore of interest, whether the PTS1 of IAT was able to locate AatB to the peroxisomes, and thus could also work as a PTS1 for a foreign protein. As shown above (results chapter 3.3.), localisation of IAT to these organelles was beneficial for penicillin production of *A. nidulans* – the acquisition of a PTS1 during evolution was therefore a conceivable specialisation event to increase the yield. Hence, also the localisation of an eGFP-AatB^{PTS1} protein fusion possessing an exchange of the C-terminal -AFE to -ANI (Fig. 32B) was analysed. The corresponding plasmid pALX-aatB^{PTS1} encoding the mutant *aatB*^{PTS1} gene was generated by inverse PCR using oligonucleotides aatB-invPCR-PTS and aatB-invPCRup (Table 4) with plasmid pALX-aatB as template. Upon re-ligation of the PCR product, plasmid pALX-aatB^{PTS1} was yielded (Table 3). By co-transformation of strain AXB4A2, strain AX_GFP-AatB^{PTS1} was generated (Table 2). All plasmids were sequenced prior to transformation (Fig. 32B, Fig. 21B for pALX-aatA) and integrated in multiple copies ectopically into the genome (data not shown).

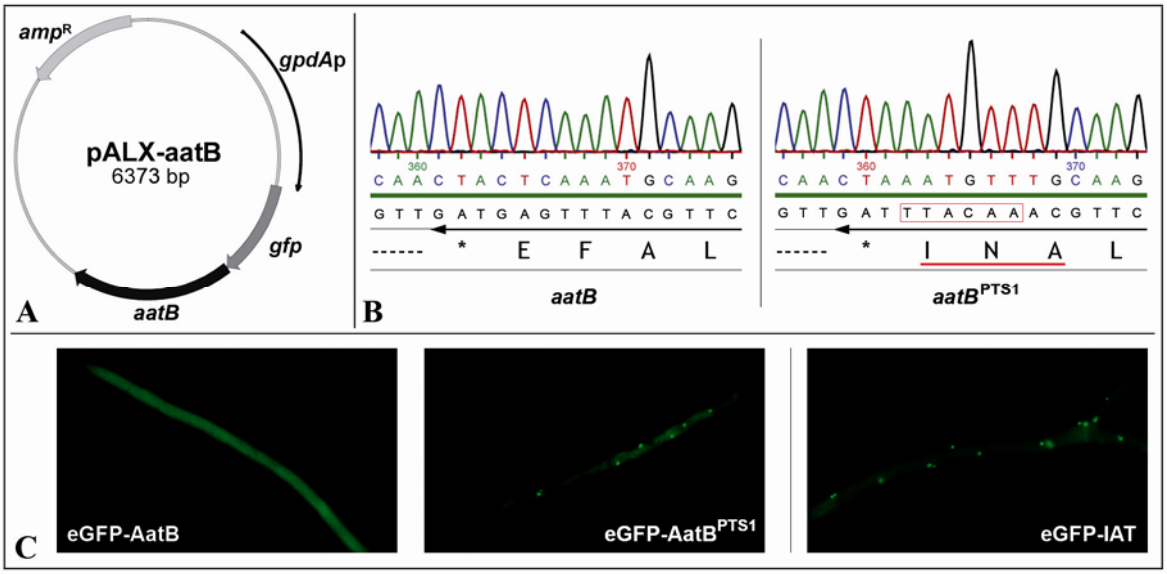

Fig. 32. Cellular localisation of *A. nidulans* AatB. (**A**) Schematic map of plasmid pALX-aatB encoding the eGFP-AatB fusion protein. (**B**) Chromatograms (complementary strands) of the *aatB* wild-type sequence (left panel), and of the -AFE to -ANI mutation resulting in introduction of the PTS1-encoding sequence of *aatA* in pALX-aatB^{PTS1} (right panel). (**C**) Localisation of the eGFP fusion proteins of *A. nidulans* strains AX_GFP-AatB and AX_GFP-AatB^{PTS1} (left panel). Strain AX_GFP-IAT was used as a control (right panel).

The localisation of the produced fusion proteins in the different strains was analysed by fluorescence microscopy (Fig. 32C). As expected from the amino acid sequence, the eGFP-AatB fusion was located in the cytoplasm whereas eGFP-IAT was transported to the peroxisomes *via* its PTS1 (-ANI; see also Fig. 21C). This PTS1 was sufficient to also locate the modified eGFP-AatB^{PTS1} fusion to peroxisomes, although residual fluorescence still remained in the cytoplasm. The cytosolic localisation of AatB was yet another hint that in *A. nidulans* the final step of penicillin biosynthesis is not restricted to peroxisomes.

In that respect it had to be ruled out that the residual penicillin production, that was observed for strain AatA$^{\Delta PTS1}$ possessing a mislocated IAT (Fig. 24B of results chapter 3.3.2.), was only due to the activity of the cytosolic AatB, and that – after all – a mislocated IAT was not functional. Therefore, strain AatA$^{\Delta PTS1}$ was crossed with the *aatB*-disruption strain AatB-disr to generate a strain with the mislocated IAT as sole source of AAT activity. Out of the progeny, a strain carrying both alleles, i.e., *aatA*$^{\Delta PTS1}$ and the *aatB* disruption, was identified by Southern blot analysis (data not shown) and designated A$^{\Delta PTS1}$/B-disr (Table 2). Penicillin production of the parental strains and strain A$^{\Delta PTS1}$/B-disr was analysed (Fig. 33). The double mutant was still able to produce penicillin. Production compared to strain AatB-disr was significantly decreased to the same extend (approximately 20 %) as that of AatA$^{\Delta PTS1}$ compared to the wild-type strain (see also Fig. 24B). Therefore, the impact of the mislocated IAT was the same for both strains and independent of AatB activity. Taken together, data confirmed that in *A. nidulans*, despite reduced activity, a mislocated IAT is still functional.

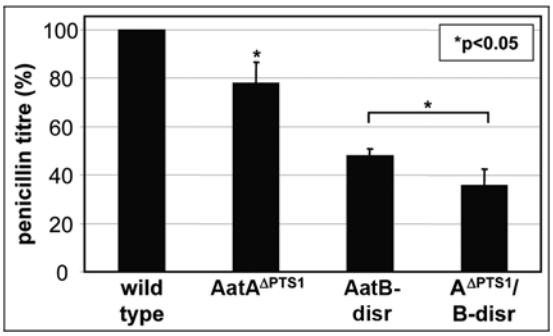

Fig. 33. Penicillin production of *A. nidulans* strain A$^{\Delta PTS1}$/B-disr. Comparison to wild-type strain TN02A21 (set 100 %) and the parental strains AatA$^{\Delta PTS1}$ and AatB-disr, respectively. Statistic significance is indicated by the p-value. Since strain A$^{\Delta PTS1}$/B-disr still produced penicillin, production of strain AatA$^{\Delta PTS1}$ was not only due to AatB.

4.3.2. Processing of IAT and AatB

Both the IAT and the AatB protein of *A. nidulans* possess the DGC motif (Fig. 27C) that in *P. chrysogenum* is important for processing of the 40 kDa pre-protein to the active IAT heterodimer consisting of the N-terminal 11 kDa and the C-terminal 29 kDa subunit (Tobin et al., 1990). With the *A. nidulans* strains producing eGFP fusions of IAT and AatB it was promising to study the processing capability of the respective proteins. Since for GFP well

established monoclonal antibodies were available (e.g., Langfelder et al., 2001; Knol et al., 2005; and Ge & Balasubramanian, 2008, used them also for fungal fusion proteins), by Western blot analysis the molecular mass of the fusion proteins could be determined. If the proteins were not processed in *A. nidulans* as suggested for the IAT by Fernández et al. (2003), a signal for a 67 kDa protein comprising eGFP (27 kDa) and the unprocessed IAT / AatB (40 kDa) was expected (Fig. 34A). Accordingly, after processing, a signal corresponding to a shorter 38 kDa protein (eGFP fused to the 11 kDa N-terminus of IAT / AatB) should be detected.

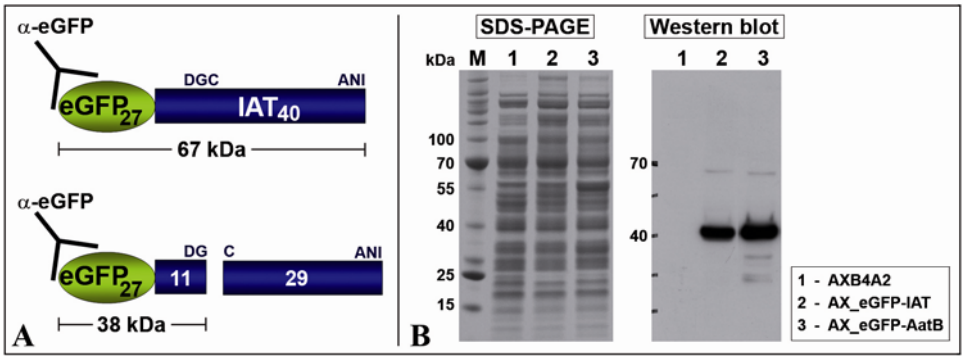

Fig. 34. Western blot analysis of eGFP-IAT and eGFP-AatB fusion proteins to elucidate processing. (**A**) Schematic depiction of the eGFP-IAT fusion protein showing expected sizes if (lower panel) or if not (upper panel) cleaved. DGC motif and PTS1 are indicated. Molecular masses for unprocessed IAT (357 aa) and AatB (345 aa) are approximately the same. Thus, similar sizes were expected for the eGFP-AatB fusion protein. (**B**) Western blot analysis of crude extracts of *A. nidulans* strains AXB4A2, AX_GFP-IAT and AX_GFP-AatB using α-GFP as primary antibody. Coomassie-stained SDS-PAGE (left panel) is shown as loading control.

A. nidulans strains AX_GFP-IAT and AX_GFP-AatB producing the respective fusion proteins (Fig. 32C) were shaken in AMM for 16 h at 37°C. Crude extracts were prepared and subjected to SDS-PAGE followed by Western blot analysis using monoclonal mouse α-GFP as primary antibody (Fig. 34B). As a negative control, crude extract of the untransformed strain AXB4A2 was used that yielded no signal. Thus, no unspecific signals were detected. For strains AX_GFP-IAT and AX_GFP-AatB clear signals confirmed the production of the fusion proteins. A very faint 70 kDa band was indicative for the full-length eGFP-IAT and eGFP-AatB protein fusions, respectively. However, for both strains a 40 kDa signal was most prominent that conceivably derived from the cleaved fusion proteins. Interestingly, for strain AX_GFP-AatB a 55 kDa band already appeared on the protein gel, but obviously this was not related to the eGFP fusion. Taken together, under the applied conditions, the *A. nidulans* IAT and AatB were most likely processed, which was in contrast to previous results obtained for the IAT (Fernández et al., 2003). Furthermore, together with the localisation experiments,

data suggested that cleavage of IAT either occurs in the peroxisomes or, if prior to translocation, that the heterodimer stays tightly associated to be able to use the PTS1 of the C-terminal subunit for transport of the whole protein. Otherwise, the eGFP protein fused to the other subunit would have been always located in the cytoplasm.

4.4. Complementation studies

Data obtained so far showed that the *aatB* gene as putative paralogue of *aatA* derived from a common ancestor gene, still plays a role in penicillin biosynthesis. To obtain insights into events that might have happened after duplication of this gene, possible evolutionary scenarios were simulated by overexpression of (i) the unmodified *aatB* gene, and (ii) the modified *aatB* gene containing the PTS1 of the *aatA* gene (*aatB*^{PTS1}) in an *aatA*-disruption strain. The encoded proteins were shown to be (i) cytosolic and (ii) mainly peroxisomal (Fig. 32C). To analyse whether these *aatB* modifications were able to complement the *aatA* disruption, the *aatB* variants were put under the control of the strong inducible *alcA* promoter of *A. nidulans* yielding plasmids pAL4-aatB(pyro) and pAL4-aatB^{PTS1}(pyro), respectively.

To generate pAL4-aatB(pyro) (Table 3; Fig 35A), the *aatB* gene was amplified using oligonucleotides aatB_up_KpnI and aatB_down_XbaI (Table 4). After sub-cloning and sequencing, the 1,588 bp DNA fragment was excised and ligated in frame with *alcA*p into the *Kpn*I/*Xba*I-linearised vector pAL4(pyro) (Table 3), that was yielded in advance by cutting the *A. fumigatus* pyridoxine biosynthesis gene as selectable marker from plasmid SM6363 using *Eco*RV and *Dra*I, and subsequent ligation into the *Eco*RV-linearised pAL4 (Table 3). Plasmid pAL4-aatB^{PTS1}(pyro) was generated by inverse PCR using oligonucleotides aatB-invPCR-PTS and aatB-invPCRup (Table 4) with pAL4-aatB(pyro) as template, followed by re-ligation of the PCR product. As a positive control, the *aatA* gene was put under the control of the *alcA* promoter. For this purpose, the *aatA* gene was amplified using oligonucleotides aatA_up_BamHI and aatA_down_BamHI (Table 4). After sub-cloning and sequencing, the 1,661 bp DNA fragment was excised and ligated in frame with *alcA*p into the *Bam*HI-linearised vector pAL4(pyro) to give plasmid pAL4(pyro)-aatA (Table 3).

Since plasmids pAL4-aatB(pyro) and pAL4-aatB^{PTS1}(pyro) possessed a 2 kbp homologous region to plasmid pbar-aatA-disrupt (pBS-SK^{+} derivatives; indicated by *hr* in Fig. 35A), they could be targeted to the *aatA*-disruption locus upon transformation of the Δ*nkuA* strain AatA-disr to generate strains A-disr_ovB and A-disr_ovB^{PTS1}, respectively (Table 2). As a positive control that was expected to fully complement the *aatA*-disruption phenotype, strain A-disr_ovA carrying the *aatA* gene under the control of the *alcA* promoter

(Table 2) was generated in a similar way by transforming strain AatA-disr with plasmid pAL4(pyro)-aatA. Integration into the genome at the *aatA*-disruption locus was confirmed by Southern blot analysis (data not shown). Inducibility of the *alcA* promoter (Waring et al., 1989), and thus of *aatB*, *aatB*^{PTS1} and *aatA* gene expression, was exemplarily shown for strain A-disr_ovB by Northern blot analysis (Fig. 35B). RNA was isolated from mycelia grown in AMM + glucose (*alcA*p-repressing conditions) for 24 h and in AMM + lactose + cyclopentanone (*alcA*p-inducing conditions) for 48 h, respectively, and hybridised to the probe indicated in Fig. 30A. Whereas with *alcA*p repressed no transcripts were detected, under *alcA*p-inducing conditions a strong signal indicated highly abundant *aatB* transcripts. The mRNA level of the endogenous *aatB* gene that was still present in the strains was too low to be detected under the applied conditions (compare to Fig. 36).

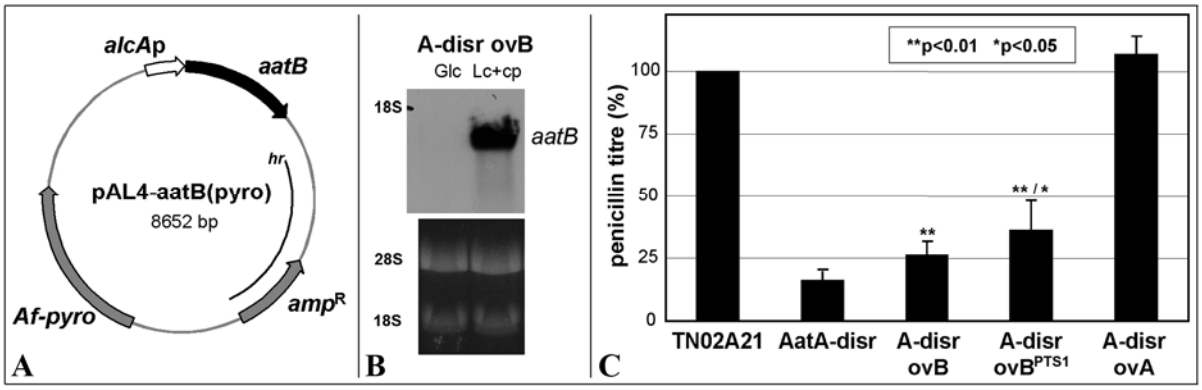

Fig. 35. Overexpression of the *aatB* gene in *A. nidulans* strain AatA-disr. (**A**) Schematic map of plasmid pAL4-aatB(pyro) carrying the *aatB* gene under the control of the inducible *alcA* promoter. *hr* marks the homologous region used to target the plasmid to the *aatA*-disruption locus of strain AatA-disr (see text). The same strategy was applied for the altered *aatB*^{PTS1} gene and the *aatA* gene, respectively. (**B**) Northern blot analysis of *aatB* transcripts in strain A-disr_ovB under *alcA*p-repressing (Glc) and *alcA*p-inducing (Lc+cp) conditions, the latter yielding high levels of *aatB* mRNA. Position of the 18S rRNA on the membrane is indicated. rRNA bands are shown as loading control. (**C**) Penicillin titres of the *aatB*- and *aatB*^{PTS1}-overexpressing strains A-disr_ovB and A-disr_ovB^{PTS1}, respectively, in comparison to the untransformed strain AatA-disr and the complemented strain A-disr_ovA overexpressing the *aatA* gene. Wild-type strain TN02A21 was set 100 %. Strains were grown in FM under *alcA*p-inducing conditions for 72 h. Statistic significance of data obtained for A-disr_ovB compared to AatA-disr and for A-disr_ovB^{PTS1} compared to AatA-disr / to A-disr_ovB, respectively, is indicated by the p-value.

Overexpression of the *aatB* gene in the *aatA*-disruption strain led to a significant increase ($p < 0.01$) of the penicillin titre (Fig. 35C) compared to the untransformed strain AatA-disr. This partial complementation was further enhanced ($p < 0.05$) by the addition of the PTS1 of IAT to AatB, leading to approximately doubled penicillin titres of strain A-disr_ovB^{PTS1} compared to strain AatA-disr ($p < 0.01$). Thus, locating AatB to the

peroxisomes had a beneficial effect. It was therefore conceivable that also during evolution a similar specialisation increased the efficiency of the enzyme. However, the peroxisomal localisation of AatB was not sufficient to completely restore wild-type levels of penicillin production, which was only observed by overexpressing the *aatA* gene in strain A-disr_ovA. Hence, further specialisation events were necessary to yield the IAT that is present today.

4.5. Expression studies

Since *aatA* and *aatB* were putative paralogues it was interesting to study whether the genes are also expressed in a similar way. The *aatA* gene was previously analysed in this regard by measuring both expression of an *aatA*p-*lacZ* fusion and IAT specific activities (Litzka et al., 1995). In the present study, time and media dependent *aatA* and *aatB* gene expression was followed and compared directly by Northern blot analysis (Fig. 36). For this purpose, wild-type strain TN02A21 was incubated in AMM and FM, and RNA that was isolated after different incubation periods was hybridised to both *aatA* and *aatB* specific probes (indicated in Figs. 25 and 30, respectively).

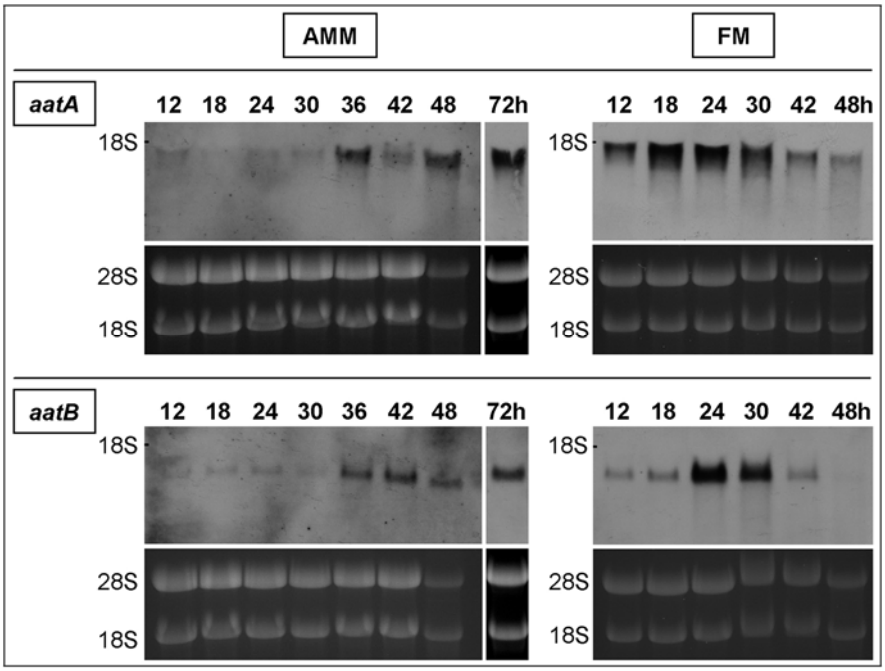

Fig. 36. Northern blot analyses of *aatA* (upper panel) and *aatB* (lower panel) transcripts. Position of the 18S rRNA on the membranes is indicated. rRNA bands are shown as loading control. As indicated above, the *aatB* transcript was smaller than the *aatA* transcript.

In AMM both genes showed similar patterns of mRNA-steady-state levels with increasing amounts after 36 h reaching a maximum after 72 h with the *aatA* mRNA in general giving a slightly stronger band (Fig. 36, left column). In FM, the mRNA-steady-state level of both genes (Fig. 36, right column) was much stronger. However, in contrast to AMM, *aatA*-mRNA levels differed from that of the *aatB* gene by increasing rapidly before decreasing after approximately 30 h, which was consistent with the results obtained for the *aatA*p-*lacZ* fusion

(Litzka et al., 1995). On the other hand, the mRNA level of the *aatB* gene increased more slowly, reaching a comparable level to *aatA* after approximately 24 h before decreasing after 30 hours. Therefore, in FM the amount of transcripts generated in total was higher for *aatA* than for *aatB* although the mRNA level of both genes decreased after 30 h. Despite these differences, the mRNA pattern of both the *aatA* and *aatB* gene in FM were similar to those of primary metabolism genes. Therefore, they were in contrast to *acvA* and *ipnA* that in FM are expressed for up to 68 h and 46 h, respectively (Brakhage et al., 1992), and thus show typical expression patterns of secondary metabolism genes (Brakhage, 1998).

4.6. Regulation of the *aatB* gene

AnCF (*A. nidulans* CCAAT binding factor; Litzka et al., 1996; Litzka et al., 1998) and AnBH1 (*A. nidulans* bHLH protein 1; Caruso et al., 2002) were previously shown to be main regulators of *aatA* gene expression. If *aatA* and *aatB* derived from a common ancestor, it was conceivable that both genes were regulated by the same transcription factors.

4.6.1. Analysis of the *A. nidulans aatB* promoter

The promoter of the *A. nidulans aatB* gene, defined as intergenic region up to the next upstream gene, i.e., AN6776.3, comprised 1,304 bp and contained both core CCAAT boxes and E-boxes as putative binding motifs for AnCF and AnBH1, respectively (Fig. 37A).

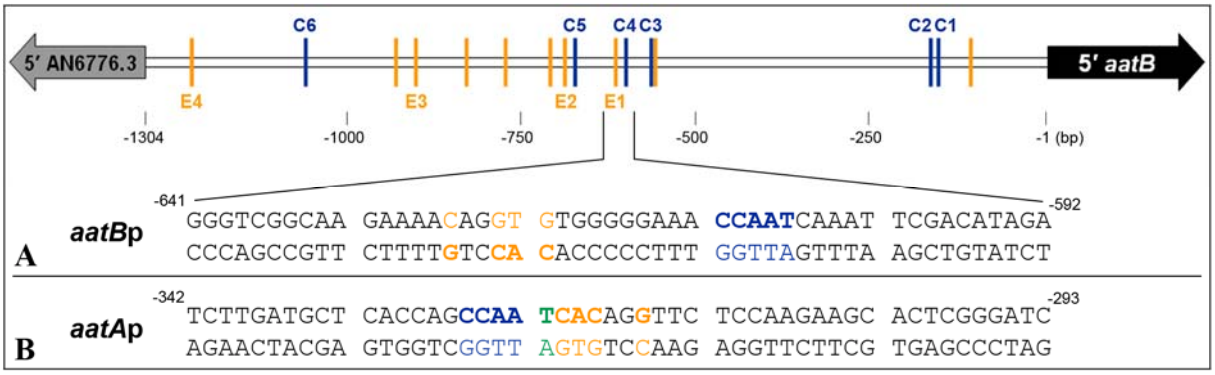

Fig. 37. Occurrence of putative binding motifs for AnCF and AnBH1 within the *aatB* promoter. (**A**) Schematic representation of the promoter region. Positions of CCAAT (C1-C6) and E-boxes (E) are indicated. Only asymmetric E-boxes are numbered. The wild-type promoter fragment (including boxes C4 and E1) used for binding studies is shown in detail. (**B**) Overlapping binding motifs for AnCF and AnBH1 within the *aatA* promoter. The thymine of the CCAAT box (green) is also essential for AnBH1 binding. The depicted DNA fragment was used as wild-type sequence for surface plasmon resonance (SPR) analysis (see below).

However, these boxes did not overlap as described for the *aatA* promoter (Fig. 37B; Caruso et al., 2002), and only a region located between -641 to -592 bp upstream of the *aatB*

gene (Fig. 37A) contained a consensus CCAAT box (Then Bergh et al., 1996; Mantovani, 1998) in close proximity to an asymmetric E-box (CACNNG). Therefore, this region was chosen for binding studies of the transcriptional regulators.

4.6.2. *In vitro* binding assays: EMSA and SPR analysis of the *aatB* promoter

To analyse binding of the transcription factors that regulate *aatA* expression to the *aatB* promoter, a DNA fragment corresponding to the in Fig. 37A indicated region was used as DNA probe for electrophoretic mobility shift assay (EMSA). This *in vitro* binding assay was previously applied to show sequence-specific binding of AnCF and AnBH1 to the *aatA* promoter by using DNA probes carrying mutations of the respective CCAAT and E-box binding motifs (Litzka et al., 1996; Caruso et al., 2002). Thus, accordingly mutated DNA fragments of the *aatB* promoter region (C_{mut} and E_{mut}, respectively; see Fig. 39B upper panel) were analysed as control to the wild-type sequence. The biotinylated DNA probes were generated by annealing oligonucleotides AatB641 and B-AatB641i (wild-type sequence), AatB641-mutC and B-AatB641-mutCi (C_{mut}), and AatB641-mutE and B-AatB641-mutEi (E_{mut}), respectively (Table 5). The recombinant purified proteins HapB, HapC and HapE, provided by Peter Hortschansky, were mixed to reconstitute the AnCF complex (Hortschansky et al., 2007). Due to the altered annotation of the *anbH1* locus in 2006 (AN7734.3, http://www.broad.mit.edu), an accordingly N-terminally extended version of AnBH1 termed AnBH1f was used. The recombinant protein was obtained from Daniel H. Scharf. Previously, it was shown that no differences between AnBH1 and AnBH1f with respect to cellular localisation and regulation of the *aatA* gene exist (Wolke, 2007).

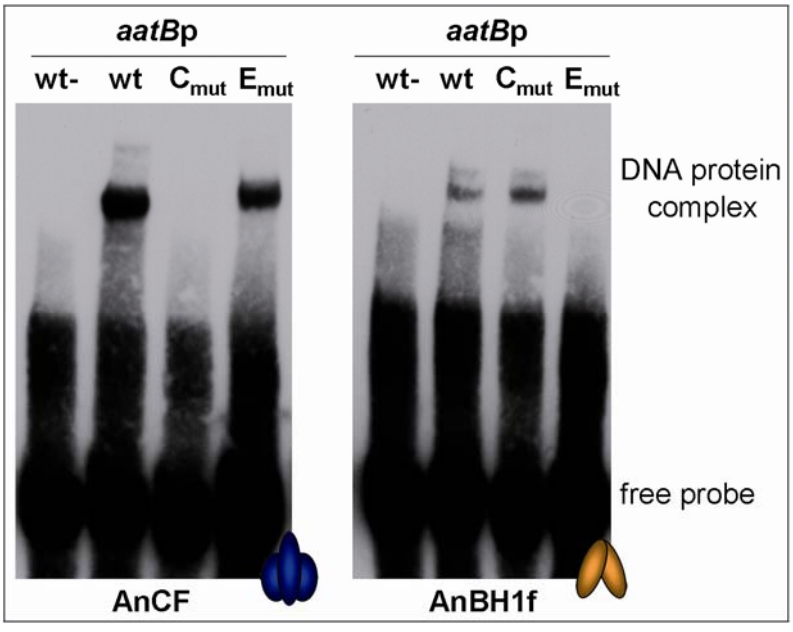

Fig. 38. EMSA analysis of binding of AnCF and AnBH1f to the *aatB* promoter. Sequences of the used DNA probes are shown in Fig. 39B. Abbreviations: wt- / wt, wild-type sequence without / with added protein; C_{mut}, mutated CCAAT box; E_{mut}, mutated E-box. Positions of the free DNA and the formed DNA protein complexes are indicated.

For clarity, the coloured symbols for AnCF and AnBH1f are used in the following.

As depicted in Fig. 38, AnCF was able to bind to the wild-type *aatB* promoter and to the E_{mut} fragment containing the mutated E-box, but not to the C_{mut} fragment comprising the mutated CCAAT box. *Vice versa,* AnBH1f did not bind to the E_{mut} fragment but was able to bind to the wild-type and to the C_{mut} fragment. These data indicated that *in vitro* binding of AnCF to the CCAAT box and of AnBH1f to the E-box located within the analysed region of the *aatB* promoter was specific and that the binding motifs, in contrast to the ones within the *aatA* promoter, were independent of each other – which was expected from the non-overlapping binding motifs. The weaker shift signal obtained for AnBH1f compared to AnCF already implied a weaker binding of this regulator.

To further study and compare kinetics and affinity of AnCF and AnBH1f interaction with both the *aatA* and *aatB* promoter in real time, surface plasmon resonance (SPR) analysis was performed. In general, this technology allows the real-time detection and monitoring of biomolecular binding events. One of the interacting molecules, in this study the DNA probe, is bound *via* the biotin-streptavidin system to the biosensor surface (sensor chip), whereas the other, i.e., the transcription factor protein, is delivered to the surface in a continuous flow through a microfluidic system. Binding to the immobilised DNA is followed by SPR, which detects the mass concentrations at the surface. This response is directly proportional to the mass of molecules that bind to the surface. However, kinetics and affinity are not calculated from association, but from dissociation of the biomolecular complex, expressed by the dissociation constant K_D. The smaller the K_D value (usually nanomolar units), the tighter the binding of the biomolecules, or the higher the affinity between DNA and protein.

As DNA probes, the 50 bp *aatA* and *aatB* promoter fragments depicted in Fig. 37 and their mutated variants, namely C_{mut} and E_{mut}, were obtained by annealing of the respective biotinylated oligonucleotides of Table 5. The different DNA probes are also listed in Fig. 39A (upper panel) for the *aatA* promoter, and in Fig. 39B (upper panel) for the *aatB* promoter, respectively. The latter were the same that were already used for the EMSA studies in Fig. 38, as were the purified proteins AnCF and AnBH1f. It was the first time that interaction of a bHLH protein and DNA was investigated by SPR analysis. As shown in the lower panels of Fig. 39A and B, the analyses enabled comparison of the binding of AnCF and AnBH1f to the wild-type sequences with binding of the regulators to the sequences containing the mutated CCAAT box (blue graphs) and the mutated E-box (orange graphs), respectively. Binding of both AnCF and AnBH1f to the *aatB* promoter (K_D values of 5.48 and 73 nM, respectively) revealed a lower affinity than binding of the regulators to the *aatA* promoter (K_D values of 1.82 and 22.7 nM, respectively). Furthermore, as already suggested by EMSA, compared to

AnCF, weaker binding of AnBH1f to the *aatB* promoter was observed. Thus, in both promoters AnCF bound with a higher affinity to the DNA than AnBH1f.

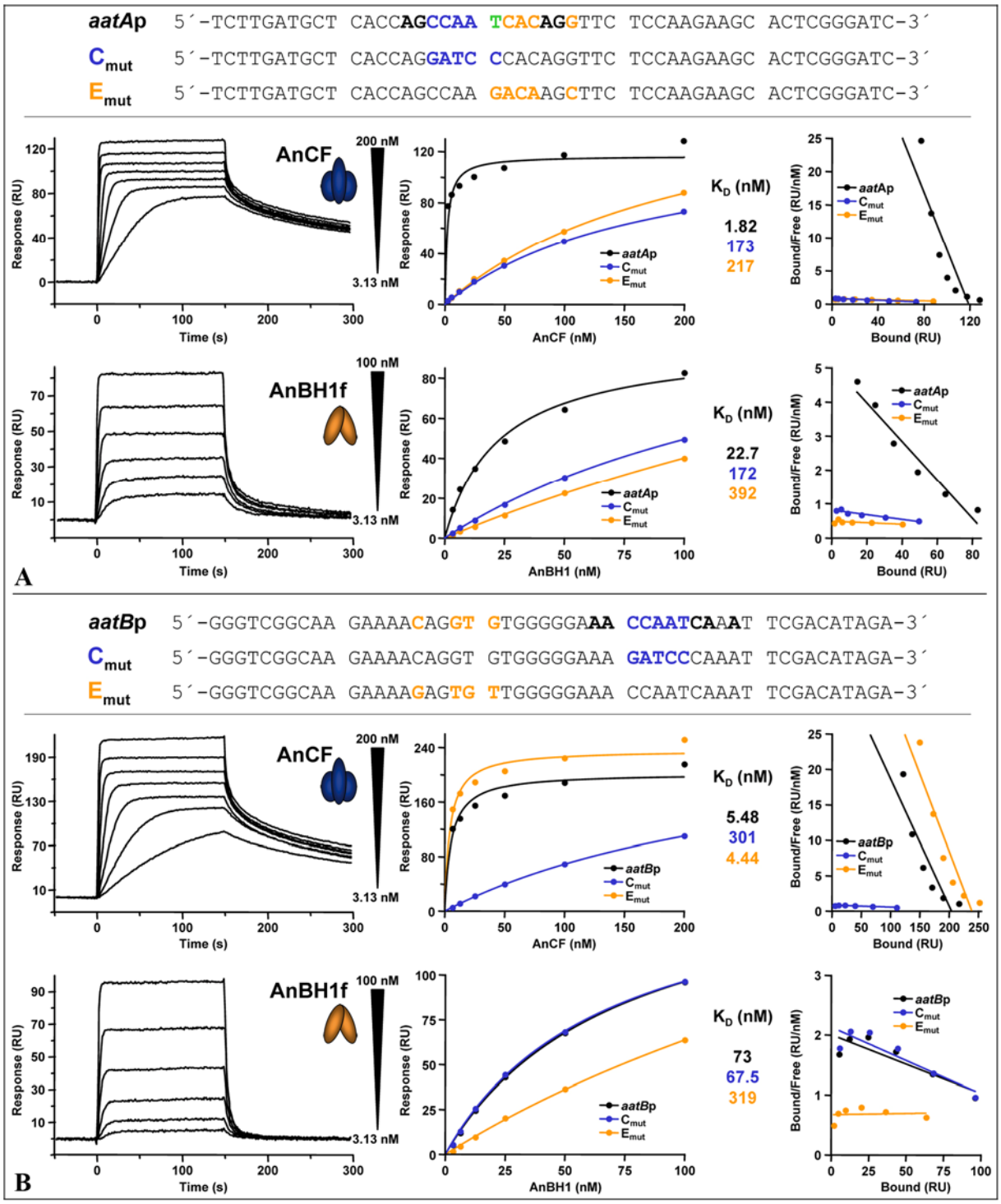

Fig. 39. Real-time *in vitro* binding of the transcription factors AnCF and AnBH1f to (A) the *aatA* and (B) the *aatB* promoter. (**Upper panels**) Wild-type sequences of the *aatA* and *aatB* promoter used as DNA probes in SPR analyses (see also Fig. 37). Below, the respective altered sequences C_{mut} and E_{mut} carrying either a mutated CCAAT box (indicated in blue) or a mutated E-box (orange). Only one strand is shown. All mutations were according to Litzka et al., 1996, and Caruso et al., 2002. Due to the overlapping nature within the *aatA* promoter (thymine marked in green), a mutation of the CCAAT box also alters the E-box and *vice versa*. (**Lower panels**)

SPR analysis of AnCF and AnBH1f binding to the promoter sequences. Left column: concentration dependent binding sensorgrams of the regulators (AnCF: 3.13, 6.25, 12.5, 25, 50, 100 and 200 nM, respectively; AnBH1f: 3.13 to 100 nM) to the biosensor-linked wild-type promoter sequences. Centre column: concentration dependent, steady-state binding of the regulators to biosensor-linked altered promoter sequences in comparison to the wild-type sequence (black). Blue: C_{mut}. Orange: E_{mut}. Right column: To show linear dependencies of the data, Scatchard plot analysis (Rosenthal, 1967) of data shown in centre column was applied. Ratios of steady-state binding values and concentration of analysed protein (RU/nM) were plotted against steady-state binding values (RU) and best-fitted by a line to display linear dependency. The steeper the negative slope of the graph the higher the binding affinity. Dissociation constant (K_D) values of AnCF and AnBH1f for binding to the different promoter sequences under the applied conditions are listed using the same colour code.

As expected, the K_D values also reflected a major difference derived from the position of the binding motifs to each other (Fig. 39). Within the *aatA* promoter, mutation of both the CCAAT box and the E-box strongly reduced DNA binding affinity of AnCF and AnBH1f to 100 times and 10 times higher K_D values, respectively, whereas within the *aatB* promoter neither the mutated CCAAT box had an impact on AnBH1f binding kinetics (67.5 *vs.* 73 nM) nor the mutated E-box on AnCF (4.44 *vs.* 5.48 nM). Therefore, within the *aatA* promoter the consensus binding motifs were overlapping while within the *aatB* promoter they were independent. Nevertheless, specificity of both AnCF and AnBH1f binding to *aatB*p was demonstrated by the increase of K_D values when the CCAAT box and the E-box, respectively, were mutated (5.48 to 301 nM for AnCF, and 73 to 319 nM for AnBH1f). Taken together, the regulators of *aatA* gene expression, AnCF and AnBH1f, were also able to bind specifically but with a lower affinity to the *aatB* promoter sequences *in vitro*.

4.6.3. Analysis of transcription factor binding *in vivo*

The observed *in vitro* interaction of the transcriptional regulators AnCF and AnBH1f with the *aatB* promoter did not necessarily imply an *in vivo* relevance similar to the one known for regulation of *aatA*. Thus, *A. nidulans hapE* and *anbH1* mutant strains were analysed for both *aatA* and *aatB* gene expression by Northern blot analysis (Fig. 40). RNA was isolated from mycelia grown in AMM for 48 h or in FM for 24 h, since under these conditions both transcripts were present at reasonable amounts in the *A. nidulans* wild-type strain TN02A21 (Fig. 36). Therefore, TN02A21 was used as reference strain. RNA was hybridised to the probe indicated in Fig. 25A (for *aatA*) and Fig. 30A (for *aatB*), respectively. Since deletion of the *anbH1* gene appeared to be lethal (Caruso et al., 2002), strain AnBH1f-GFP overproducing a functional eGFP fusion of the N-terminally extended AnBH1f (*ov_anbH1f* in Fig. 40; Table 2) was used to study the effect of highly abundant AnBH1f on

aatA and *aatB* gene expression. As shown in Fig. 40 (upper panel left), in AMM absence of a functional AnCF in strain ΔE-89 (carrying a deletion of the AnCF subunit-encoding *hapE* gene; Table 2) or abundance of AnBH1f led to low levels of *aatA* mRNA. These results were consistent with AnCF acting as an activator and AnBH1 as a repressor of *aatA* expression in AMM (Litzka et al., 1996; Caruso et al., 2002). Interestingly, in AMM, the *aatB* mRNA steady-state level was obviously not altered in the mutant strains (Fig. 40, lower panel left) indicating only a minor role for AnCF and AnBH1f under these conditions.

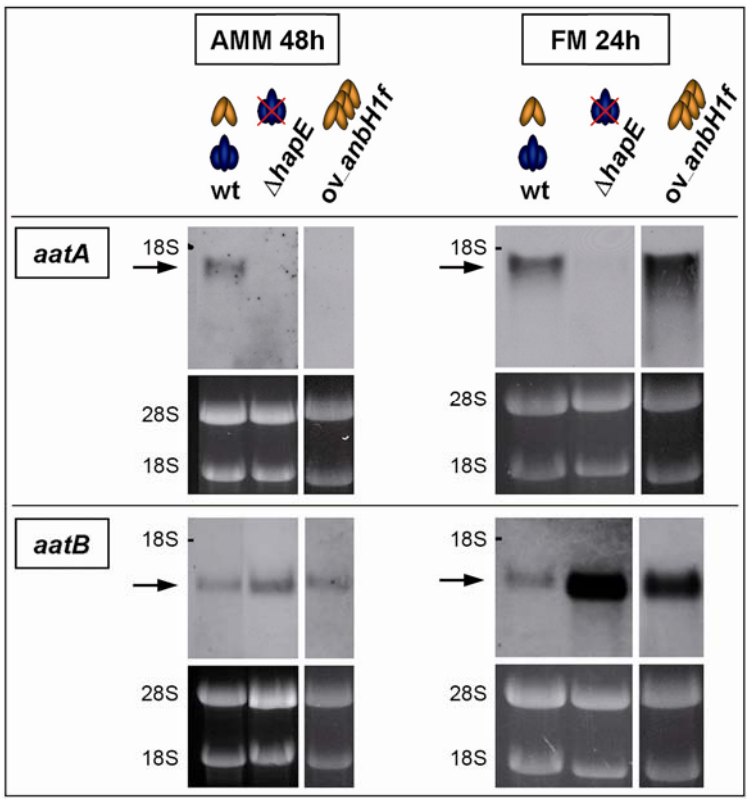

Fig. 40. Northern blot analyses of *aatA* (upper panel) and *aatB* (lower panel) transcripts in different *A. nidulans* mutant strains. Respective signals are indicated by arrows. Incubation of the strains in AMM (left panel) and FM (right panel). Position of the 18S rRNA on the membranes is marked, and rRNA bands are shown as loading control. Abbreviations: wt, *A. nidulans* wild-type strain TN02A21; Δ*hapE*, *A. nidulans* strain ΔE-89: no formation of the AnCF complex; *ov_anbH1f*, *A. nidulans* strain AnBH1f-GFP: overproduction of a functional AnBH1f-eGFP fusion.

In FM, *aatA* gene expression again was low in the absence of a functional AnCF (Fig. 40, upper panel right) but mRNA levels were slightly increased by overexpression of *anbH1f* which was in contrast to the effect of AnBH1f in AMM. It was therefore conceivable that the regulator had a moderately positive effect on *aatA* gene expression during the early phase of a fermentation run. A previous study indicated that an obvious repressing effect of AnBH1 in FM was detectable only after longer incubation periods, i.e., 48 h (Herrmann et al., 2006). Whereas both absence of a functional AnCF and abundance of AnBH1f had no clear influence on *aatB*-mRNA levels in AMM, both modifications caused a strong increase of *aatB* transcripts in FM (Fig. 40, lower panel right). Under these conditions, AnCF appeared to be a repressor and AnBH1f an activator of *aatB* gene expression, which was different from the effect of AnCF and AnBH1f on *aatA* gene expression. However, the data shown here indicate that *in vitro* binding of AnCF and AnBH1f to the *aatB* promoter also had significance

in vivo, and thus, confirmed the importance of the regulators for expression of both genes *aatA* and *aatB* in *A. nidulans*. Moreover, this obviously co-regulated expression was indicative for a common evolutionary path of the two genes and, in addition, suggested a hypothesis concerning the origin of the transcriptional network regulating the penicillin biosynthesis gene cluster.

4.6.4. Analysis of binding sites in homologous genes of other fungi

As outlined in Fig. 28 and Table 7, putative homologues of the *aatB* gene were widely distributed within the Aspergilli, whereas *aatA* homologues were only found in association with the respective *acvA* and *ipnA* homologues and thus, only in penicillin producing fungi.

Table 7. Occurrence of the penicillin biosynthesis gene cluster, of *aatA* and *aatB* homologues, and of putative transcription factor binding sites within the respective promoters in Aspergilli, *Neosartorya fischeri* and *Penicillium chrysogenum*. *A. terreus* has two putative *aatB* homologues (Fig. 28); *aatA* homologues occur only in association with the gene cluster. Locus IDs of the genes are indicated (*Pc-aatB*: personal communication M. van den Berg). CCAAT: CCAAT box as binding motif for AnCF; CACNNG: asymmetric E-box as binding motif for AnBH1. For *aatA*, the promoter was defined as the intergenic region between the *ipnA* and *aatA* homologues, for putative *aatB* homologues as the intergenic region up to the next upstream gene (but at least 1,000 bp). For *aatB* of *A. nidulans* (AN6775.3) see also Fig. 37.

	penicillin cluster	*aatA* homologues	CCAAT	CACNNG	(putative) *aatB* homologues	CCAAT	CACNNG
A. nidulans	+	AN2623.3	**1**	**11**	AN6775.3	**6**	**4**
A. oryzae	+	AO090038000545	**6**	**3**	AO090005000709	**4**	**2**
A. flavus	+	AFL2G_07877	**5**	**4**	AFL2G_00699	-	**2**
A. clavatus	-	-	-	-	ACLA_040440	**2**	**4**
A. terreus	-	-	-	-	ATEG_07911	**2**	**5**
					ATEG_04134	-	**3**
A. fumigatus	-	-	-	-	Afu3g12620	**1**	**7**
A. niger	-	-	-	-	An02g08570	**4**	**6**
N. fischeri	-	-	-	-	NFIA_064690	**1**	**4**
P. chrysog.	+	ABA70584	**4**	**12**	*Pc-aatB*	**2**	**7**

Analysis of the transcriptional regulation of both genes in *A. nidulans* indicated, that the ancestral gene of which *aatA* and *aatB* most likely derived from, was also regulated by AnCF and AnBH1, and that gene duplication included the regulatory sequences. In addition, previous reports (Brakhage et al., 1999; Caruso et al., 2002) and ongoing database analyses revealed that (putative) homologues of the AnCF subunits and of AnBH1 were also present in

other Aspergilli. Therefore, it was interesting to analyse the *aatA* and putative *aatB* homologues of these filamentous fungi for the occurrence of respective regulatory motifs within their promoters. Table 7 summarises the *in silico* studies revealing that putative AnCF and AnBH1 binding sites were present. Asymmetric E-boxes could be found in all analysed promoters, CCAAT boxes were only missing in the promoter of the putative *aatB* homologue of *A. flavus* and in one of the putative *aatB* homologues of *A. terreus*. However, whether these motifs are relicts of evolution or do truly have a relevance in regulation of the genes, remains the objective for further studies.

4.7. Functional characterisation of AatB – first investigations

Since *aatB*-like genes were widely distributed in fungi that do not produce penicillin, the encoded protein, if synthesised at all as shown for *A. nidulans*, had to fulfil besides converting IPN additional functions– also in the producing organisms. An *A. nidulans aatB*-disruption strain was viable; thus, the gene is not essential for the direct survival of the fungus. Since AatB possessed an acyl-CoA transferase domain (Fig. 27) necessary for catalysing the final step of penicillin biosynthesis, it was conceivable that the protein either played a role in fatty acid metabolism or in detoxification processes, e.g., to get rid of toxic compounds like phenylacetic acid (PA), which is one theory about the purpose of penicillin production in general. However, growth of the *aatB*-disruption strains AatB-disr and AatA/B-disr (Table 3) on different short and long-chain fatty acids as sole carbon sources was not impaired in comparison to the wild-type strain TN02A21 (data not shown). Therefore, disruption of *aatB* had no influence on fatty acid metabolism. Interestingly, these strains were also able to utilise benzoic acid (BA) and PA at non-toxic concentrations of 10 mM (Mingot et al., 1999) as sole carbon sources. In food industry, BA is used as organic acid food preservative (Piper et al., 2001). The toxicity of PA most likely derives from lowering the internal pH and dissipation of transmembrane pH gradients through passive diffusion, inhibiting processes like hyphal tip growth and uptake of solutes (Hillenga et al., 1995).

If AatB was involved in detoxifying processes, disruption strains should be more susceptible to these weak organic acids. Therefore, AatB-disr and AatA/B-disr were exposed to toxic concentrations of the substances, which were found to be 1.0 % (w/v) for BA and 1.5 % (w/v) for PA, i.e., 82 mM and 110 mM, respectively. Glucose was used as main carbon source, and outgrowth of serially diluted conidia was compared to that of the wild-type strain TN02A21 and of the *aatA*-disruption mutant AatA-disr. Surprisingly, the *aatB*-disruption strains were found to be more resistant to BA and PA than these control strains (Fig. 41),

which was not observed for 1.5 % (w/v; i.e., 99 mM) phenoxyacetic acid (POA), maybe due to a lower accessibility of this compound. In addition, sporulation of the *aatB*-disruption mutants appeared to be less restricted in the presence of PA. Interestingly, in all analysed aspects, the phenotype of the *aatA*-disruption mutant was similar to that of the wild type.

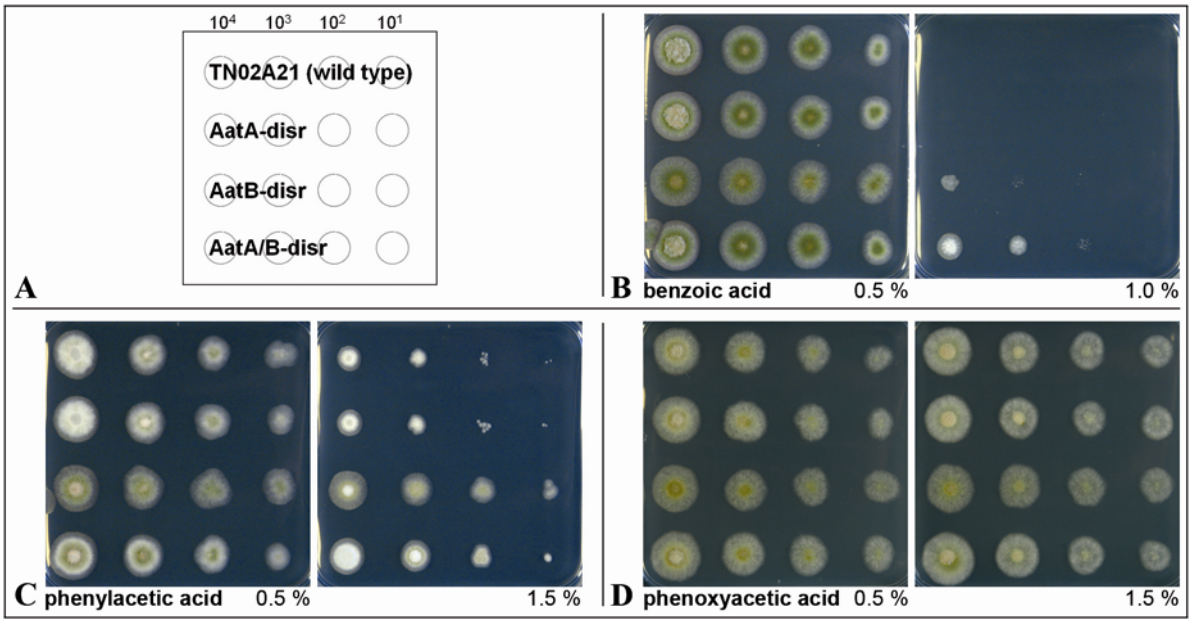

Fig. 41. Sensitivity of *A. nidulans aatB*-disruption strains AatB-disr and AatA/B-disr to weak organic acids. Strains TN02A21 (wild type) and AatA-disr (*aatA* disruption) were used as controls. (**A**) Schematic drawing of the test. 10^4, 10^3, 10^2 and 10^1 conidia in a volume of 5 µl were spotted on AMM agar square plates supplemented with the indicated concentrations of benzoic acid (**B**), phenylacetic acid (**C**) and phenoxyacetic acid (**D**). 0.8 % (w/v) of glucose was used as main carbon source.

The increased resistance to PA was also observed for in liquid media. In both AMM and FM (not shown), strain AatB-disr was able to grow at elevated concentrations of PA compared to the wild type (Fig. 42). Interestingly, with addition of PA to the medium, fungi showed a hypobranching phenotype (enlargements in Fig. 42), i.e., the mycelia hardly formed branches irrespective of the genotype. Furthermore, septa formation was in neither strain affected, as analysed by staining of the fungal cell wall with calcofluor white (Pringle, 1991; data not shown).

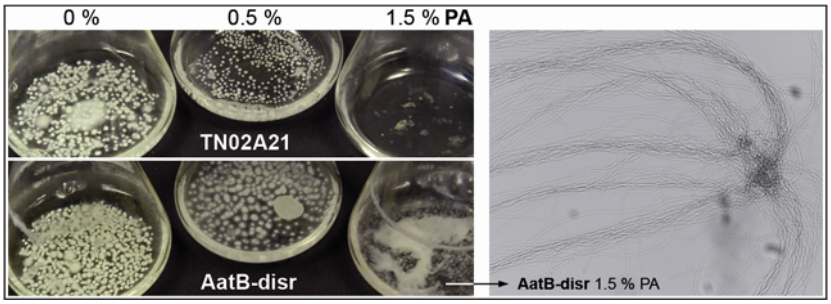

Fig. 42. Growth of *A. nidulans* strains TN02A21 (upper panel) and AatB-disr (lower panel) in the presence of phenylacetic acid (PA) in AMM. Strains were incubated for 48 h at 37°C with the indicated concentration of

PA. The enlargement shows the hypobranching phenotype of strain AatB-disr in the presence of 1.5 % (w/v) PA analysed by light microscopy (100 × magnification). This was also observed for the wild-type strain TN02A21 at the 0.5 % (w/v) concentration of PA (not shown).

As a consequence of much better growth, strain AatB-disr also produced more penicillin compared to the wild-type strain in the presence of elevated concentrations of PA, i.e., 0.5 % (w/v). However, this overall penicillin production was still less than that one observed for the wild-type strain under standard fermentation conditions, i.e., with 0.05 % (w/v) PA (Fig. 43) – most certainly due to the effect of an *aatB*-disruption on penicillin biosynthesis in general.

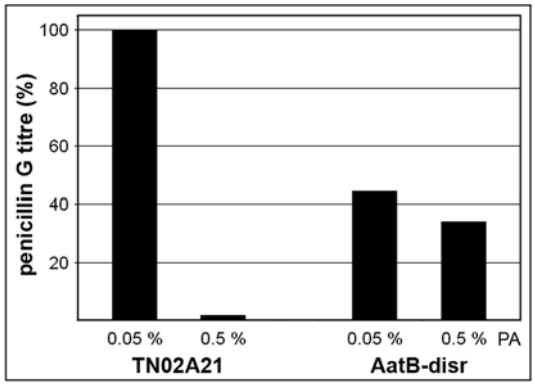

Fig. 43. Penicillin G production of *A. nidulans* strains TN02A21 and AatB-disr at standard and elevated concentrations of phenylacetic acid (PA). PA was at the same time used as substrate for the final step of penicillin G biosynthesis. The FM culture supernatants were analysed by bioassay, and wild-type penicillin production under standard conditions, i.e., with 0.05 % (w/v) PA, was set 100 %. The decrease of penicillin production of the wild type TN02A21 in the presence of 0.5 % (w/v) PA was likely due to its strong growth defect caused by the toxic compound, that was not observed for the *aatB*-disruption strain AatB-disr.

Thus, the *aatB* disruption enabled the strain to produce penicillin in the presence of usually toxic concentrations of PA. The mechanism behind this higher resistance to PA, and also to BA, based on the lack of AatB requires further investigations.

DISCUSSION

Due to its high specificity, the low toxicity and the resulting clinical significance, penicillin (along with other β-lactams) became one of the most important antibiotics. Consequently, its biosynthesis – involving three genes that are organised in a cluster – is probably the best understood pathway in fungal secondary metabolism. However, further questions need to be addressed for an entire elucidation of the pathway. This includes both regulation, particularly *via* so far unknown external signals, and evolution of the gene cluster. It was therefore the aim of this work to obtain novel insights into these aspects of penicillin biosynthesis in *Aspergillus nidulans*.

1. Regulation of penicillin biosynthesis: new players in signal cascades

With PacC, AnCF and AnBH1, important regulators that directly interact with *cis*-acting sequences of the *A. nidulans* penicillin biosynthesis genes, have been identified over the last decade (summarised in Brakhage et al., 2004). However, at the same time, elucidation of further upstream acting elements of the signal transduction cascades – or even of the triggering environmental signals themselves – was not that successful. In this work, two different strategies to identify and analyse the connecting parts of a signalling pathway between an environmental signal and the corresponding *trans*-acting factor were applied, each starting at one end of a cascade. In the first case, the external signal light led to the observation that the light-dependent developmental regulator VeA, directly or indirectly, acts as a repressor of penicillin biosynthesis. In the second case, a reciprocal approach started with the transcriptional regulator AnBH1, whose amino acid sequence analysis resulted in the finding that a central protein kinase, PkcA, is involved in regulation of penicillin production.

1.1. Velvet A (VeA)

VeA was previously shown to mediate a developmental light response (Käfer, 1965; Mooney & Yager, 1990; Yager, 1992). Strains containing a wild-type allele of the *veA* gene display in light a reduced and delayed formation of cleistothecia and the fungus develops asexually, whereas in the dark fungal development is directed towards that sexual stage. Under conditions inducing sexual development, the *veA* deletion (Δ*veA*) strain is unable to develop sexual structures (Kim et al., 2002), indicating that *veA* is required for cleistothecium and ascospore formation. Until now, the molecular mechanism by which *veA* regulates sexual development is unknown, as VeA does not show similarity with any known protein that could

indicate its function (Calvo et al., 2004). Previously, by measuring the mRNA steady state level of the penicillin biosynthesis gene *acvA*, Kato et al. (2003) showed that mRNA was only detectable in *veA* wild-type strains but not in *veA* deletion mutants, thus suggesting that VeA was an activator of penicillin biosynthesis. This result was rather surprising because most of the studies dealing with the regulation of the penicillin biosynthesis genes in *A. nidulans* (e.g., MacCabe et al., 1990; MacCabe et al., 1991; Brakhage et al., 1992; Brakhage & Van den Brulle, 1995; Pérez-Esteban et al., 1995; Mingot et al., 1999) were carried out in *veA1* strains, i.e., in strains possessing an N-terminally truncated version of VeA (VeA1; Fig. 4). These strains lack nuclear localisation of the protein (Stinnett et al., 2007), but despite the mislocalisation of the alleged activator they produce considerable amounts of penicillin. To elucidate this obvious contradiction, the influence of VeA on penicillin biosynthesis was analysed by applying two different promoters for overproduction of VeA, i.e., the *alcA* and *niiA* promoter derived from the *A. nidulans* alcohol dehydrogenase and nitrite reductase gene, respectively. These promoters were fused in frame to the *veA* gene to give inducible *alcA*p-*veA* (Fig. 5A) and *niiA*p-*veA* constructs (Fig. 10A). Strains carrying multiple copies of one of these overexpression cassettes ectopically integrated into the genome, and, in addition, encoding fusions of the penicillin biosynthesis genes *acvA* and *ipnA* to two different reporter genes, i.e., *uidA* and *lacZ*, respectively, were analysed with respect to reporter gene expression and penicillin production. Moreover, the influence of two different *veA* alleles on penicillin production was tested, i.e., *veA1* and a deletion of the *veA* gene. The functionality of VeA produced by the different promoters, i.e., *alcA*p and *niiA*p, was proven by analysing the conidiation of *veA*-overexpressing strains. Formation of asexual spores by these strains was regulated by induction or repression of the *veA*-controlling promoter (Figs. 6, 9B, and 10C; Kim et al., 2002). Additionally, in strain ΔveA a-veA the production of the dark brown pigment typical for *veA* deletion strains was absent under *alcA*p-inducing conditions indicating a complementation of this phenotype by *veA* (Fig. 9B; Krappmann et al., 2005). Therefore, these findings showed that *veA* overexpression could complement the *veA(1)* mutants and, thus, the gene and the respective protein were not only transcribed and synthesised, respectively, but also functional under the applied conditions.

In all genetic backgrounds used and under all different combinations, overexpression of *veA* led to a reduction of both the penicillin titre and the expression of the *acvA*p-*uidA* gene fusion. This action of VeA produced by inducible promoters was only slightly dependent of whether cultures were incubated in light or in darkness (Fig. 8 for *alcA*p, not shown for *niiA*p; see below). The expression of the *ipnA*p-*lacZ* gene fusion was only moderately affected in the

*alcA*p-*veA* system indicating that the repressive effect on *ipnA* is less prominent. Taken together, these data clearly indicate that VeA acts as a repressor of both the penicillin biosynthesis gene *acvA* and the penicillin production. These results well correlate with the *acvA*-encoded ACVS being the rate limiting enzyme of the penicillin biosynthesis pathway under standard fermentation conditions: overexpression of *acvA* led to drastically increased production of penicillin (Kennedy & Turner, 1996), while overexpression of *ipnA* and *aatA* did not (Fernández-Cañón & Peñalva, 1995). Therefore, it is likely that the repressive effect of VeA on penicillin biosynthesis is due to the repression of *acvA* expression.

The apparent discrepancy of these findings compared to the results of Kato et al. (2003) may be explained by the use of different methods to measure *acvA* gene expression. Whereas Kato et al. used semi-quantitative RT-PCR with cultures grown on solid YGT media (containing yeast extract, glucose and trace elements), in this work the specific activity of the heterologous reporter β-GLU was mainly measured in liquid FM. Under the applied conditions, Kato et al. could detect the *acvA* transcripts only in the strain encoding a wild-type *veA* gene, but not in the deletion strain; the *veA1* mutant was not tested. Compared to a positive control containing genomic DNA as a template, the amount of transcript in the wild-type strain was very low which might indicate a poor expression of the penicillin biosynthesis genes, when the strains were incubated on solid YGT. The glucose used as a carbon source might have exerted an additional repressing effect. Therefore, the expression level of the *acvA* gene might have been not sufficient to detect clear differences in the *veA* mutant and the wild type for both penicillin biosynthesis gene expression and penicillin production. This could easily lead to misinterpretations. Alternatively, VeA could have a regulatory role in mRNA stability, but this is rather unlikely because it would not explain the differences concerning the penicillin titres. Also, the apparently contradictory results might suggest a dual activity of VeA as repressor and activator depending on the abundance of the protein in the cell. In that case, at physiological levels VeA would have a positive effect on *acvA* gene expression and thus on penicillin biosynthesis, whereas when *veA* is under the control of a strong inducible promoter like *alcA*p or *niiA*p it might have oligomerised and act as a repressor on both processes. Based on very recent data showing that VeA exerts its function in different physiological processes of *A. nidulans* by forming the heterotrimeric *velvet* complex VelB/VeA/LaeA (Fig. 44; Bayram et al., 2008b), Calvo (2008) suggested the need of a finely balanced stoichiometry of VeA with other factors that requires a correct dosage of the protein components in order to form an activating functional complex. This means that alteration of VeA-protein levels (like the increase in the present study) could have a detrimental effect on

penicillin biosynthesis (Calvo, 2008). Again, this interpretation seems unlikely because the overproduced VeA, in terms of sporulation and pigment formation, complemented both the *veA1* and *veA* mutant to the wild type. However, for other signal transduction cascades involving VeA, a disadvantageous influence of abundant VeA cannot be ruled out.

In the presence of light, the repressing effect of VeA appeared to be less prominent (Fig. 8D-F), which is consistent with the dependence of nuclear localisation of VeA on light: the current model suggests that with light VeA is mostly retained in the cytoplasm and thus, is not able to exert its regulating function (Stinnett et al., 2007; Bayram et al., 2008b). This model links penicillin production to light and therefore to a not previously known external stimulus. As already indicated in the results chapter, in submerse FM cultures the equal availability of light for all mycelia was most likely not assured, leading to only a moderate effect. However, together with the complementing capability of VeA overproduction, it was indicative for the functionality of the system. Nevertheless, the striking observation that in *Acremonium chrysogenum* the VeA homologue AcVEA is essential for normal production of the β-lactam antibiotic cephalosporin C and thus, in this organism does not act as repressor of a secondary metabolite (Dreyer et al., 2007), requires further investigation concerning the different roles of VeA in different species that was already suggested by other studies (e.g., Li et al., 2006, for *Fusarium verticillioides FvVE1*, and Bayram et al., 2008c, for *Neurospora crassa ve-1*). Furthermore, it is not unusual that a single protein has opposite roles in regulating the biosynthesis of secondary metabolites in the same organism, as it would be the case for VeA repressing penicillin and activating sterigmatocystin (ST) biosynthesis in *A. nidulans*, respectively, the latter suggested by Kato et al. (2003). Such a role was previously described for FadA, the α-subunit of a heterotrimeric G-protein, which is involved in both penicillin and ST biosynthesis (Tag et al., 2000).

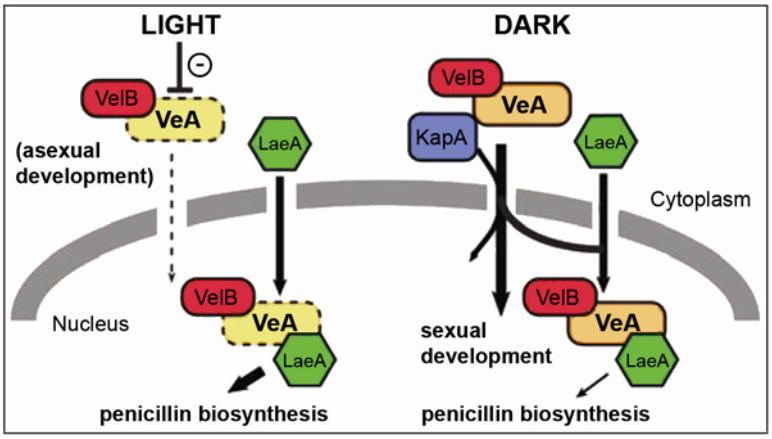

Fig. 44. Proposed model of the light-dependent regulation of the *velvet* complex in *A. nidulans* and its role in penicillin production (adapted from Bayram et al., 2008b). (Light) VeA is located mainly in the cytoplasm, VelB as developmental regulator supports asexual spore formation, and LaeA as global regulator of secondary metabolism shows low activity. Dashed lines indicate the decreased amount of VeA that is present in the cell and impaired nuclear transport of VeA under light conditions, de-repressing penicillin production. (Dark) An increased

amount of VeA is imported into the nucleus by the α-importin KapA and, in addition, supports the nuclear transport of VelB. Both higher abundance of VeA and its nuclear localisation promotes sexual development, and according to the results obtained in this work also has a repressing effect on penicillin biosynthesis.

The model presented by Bayram et al. (2008b) proposes VeA as part of a complex – the *velvet* complex – that regulates different physiological processes in *A. nidulans* in a light-dependent manner (Fig. 44). However, the up- and downstream acting signal cascades leading to and from this complex remain to be elucidated; i.e., the questions of how a light signal is transmitted to VeA and how VeA mediates its repressing effect on the *acvA* gene promoter. The present study showed that the latter is not *via* the putative repressor binding site located within the *acvA* promoter (Litzka et al., 1998), since overexpression of *veA* in strain ΔCCA-G a-veA (carrying a deletion of the respective binding site) still reduced *acvA*p-*uidA* gene expression. It is conceivable that the global regulator LaeA, a putative methyl transferase that is part of the *velvet* complex (Fig. 44) and whose deletion was shown to alter transcription levels of secondary metabolite gene clusters in *A. nidulans* (Bok et al., 2006), might play a role in VeA-mediated regulation. Concerning light sensing, three possible upstream factors have been identified so far: a phytochrome, FphA (Blumenstein et al., 2005); two blue-light receptor systems, LreA and LreB (Purschwitz et al., 2008); and a cryptochrome, CryA (Bayram et al., 2008a). It is speculated that due to this abundance different protein interactions or protein complexes occur at different times in the cell (Fischer, 2008).

Until now, the physiological meaning of the VeA- and therefore light-dependent regulation of the penicillin biosynthesis is unclear. Similarly, there are other examples of light-regulated metabolic processes that are difficult to interpret, as there is no obvious connection between light and the respective process. For example, in *Trichoderma reesei*, it is reported that expression of *env1*, a gene encoding the PAS/LOV domain protein Envoy involved in cellulase gene expression, is light dependent (Schmoll et al., 2005). The influence of light on cellulase gene expression well correlates with its co-regulation with sporulation, because cellulases are also found on the conidia of *T. reesei*, whose development in turn is stimulated by light. It is therefore possible that the light-induced activation of penicillin biosynthesis in *A. nidulans via* absence of and therefore the lack of VeA repression under light might be a product of co-regulation with sporulation. Because colonisation of new habitats in general takes place on the surface of the soil and therefore under the exposure of light, it may be advantageous for the conidia and the developing mycelia to produce penicillin as protection against other soil microorganisms. Another important factor could be the integrity of the cell wall which in *Fusarium verticillioides*, another filamentous fungus, seems

to be influenced by the VeA homologue FvVE1. Li et al. (2006) reported that the phenotypic alterations obtained in *FvVE1* deletion strains are most likely related to cell wall defects. If *veA* had a similar role in *A. nidulans*, then, without VeA, the cell wall would be more sensitive to attacks, e.g., by other soil microorganisms like streptomycetes, and therefore an increase of penicillin production as a self-defensive compound would be reasonable.

Extensive research during the past few years has linked the VeA protein – which is restricted to the fungal kingdom and does not show sequence similarities with any protein of known function – to diverse cellular processes (reviewed in Calvo, 2008). It is hypothesised that VeA could act as a scaffold protein that integrates external stimuli with a nuclear response by an orchestrated action with other proteins leading to adaptation processes with respect to either morphology (sexual *vs.* asexual development) or metabolism (regulation of secondary metabolism). The present study not only contributed to these findings, but extended our knowledge of regulatory pathways of penicillin biosynthesis in *A. nidulans*, and led to the identification of a new external signal, i.e., light.

1.2. Protein kinase C A (PkcA)

The involvement of VeA in regulation of penicillin biosynthesis was a rather coincidental finding since the protein is not part of a classical signal transduction pathway like those including, e.g., G-proteins and MAP kinases. These pathways are naturally the first to be investigated when searching for putative regulators. For example revealed previous work that FadA, the α-subunit of a heterotrimeric G-protein, is involved in the regulation of penicillin biosynthesis. In an *A. nidulans* strain containing a constitutively active FadA (*fadA*^{G42R}), an increased steady state level of *ipnA* mRNA and concomitantly increased penicillin titres were observed. Hence, FadA appears to be a member of a signal transduction cascade activating the penicillin biosynthesis in *A. nidulans* (Tag et al., 2000). However, the transcription factor target of this putatively classical pathway has not been identified yet.

Since almost all signalling processes employ specific protein kinases (Heinisch, 2005) and, in addition, bHLH transcription factors were shown to be phosphorylated (Littlewood & Evan, 1994), analysis of the bHLH protein AnBH1 as regulator of *aatA* gene expression was an appropriate method to identify signal transduction cascades regulating penicillin biosynthesis in *A. nidulans*. 2D gel electrophoresis experiments indicated that AnBH1 can be phosphorylated by a heterologous host (Fig. 12), and *in silico* analysis of its amino acid sequence revealed a conserved protein kinase C (PKC) target sequence with a high probability to be used (Fig. 13). Microscopic analyses of an AnBH1-eGFP fusion exposed to the PKC

inhibitor calphostin C verified the influence of a PKC on nuclear localisation of the transcriptional regulator (Fig. 14), which could be restricted by *pkcA*^{antisense} experiments to the action of PKC A (PkcA). Reduced expression of *pkcA* resulting from production of *pkcA*^{antisense} RNA led to reduced maintenance of AnBH1-eGFP in the nucleus and, furthermore, to an accumulation of the protein in the cytoplasm (Fig. 15). Therefore, PkcA was a direct target of calphostin C, because both addition of the inhibitor and production of *pkcA*^{antisense} RNA led to a decrease of AnBH1-eGFP in the nucleus and, furthermore, to an increase of this protein in the cytoplasm.

The observation that under *pkcA*^{antisense}-inducing conditions most, but not all AnBH1-eGFP molecules were located in the cytoplasm can be explained by the fact that, by using the antisense approach, expression of a given gene usually is not completely turned off but can only be reduced (Zadra et al., 2000). The possible dimension of such a knock down was previously quantified by measuring *pepB* mRNA reduction rates ranging from 10 % to 70 % when *pepB*^{antisense} RNA was produced from the *A. nidulans gpdA* promoter in *Aspergillus awamori* (Moralejo et al., 2002). Therefore, the level of antisense RNA may not be sufficient to titrate out completely the respective mRNA. Nevertheless, when the gene is essential – which is likely for *pkcA* – an antisense approach still allows its functional characterisation, especially when formation of the antisense RNA is inducible (Zadra et al., 2000).

Data strongly suggest that PkcA is involved in the nuclear entry or maintenance of AnBH1 in the nucleus, most likely by phosphorylation of AnBH1, either directly or by activating other kinases that phosphorylate AnBH1. That this interaction directly correlates with expression of *aatA*, the target gene of AnBH1, was shown by the increase of *aatA*p-*lacZ* reporter gene expression under *pkcA*^{antisense}-inducing conditions (Herrmann et al., 2006). Interestingly, this higher expression of the third penicillin biosynthesis gene led to a reduced penicillin titre, an observation that was explained by the penicillin amidase activity of the encoded IAT enzyme, which represents the reversion of the biosynthetic 6-APA acyltransferase activity (Alvarez et al., 1993; see also introduction chapter 2.2.). The increase of this enzyme activity by overproduction of IAT was assumed to lead to less active penicillins or to an increase in 6-APA which has no significant antibiotic activity. In addition, it was conceivable that PkcA directly acts on other elements of the penicillin biosynthesis.

Taken together, by the reciprocal approach of the present study – starting from the transcription factor and going upwards – the protein kinase C PkcA of *A. nidulans* was found to be involved in AnBH1 regulation. This links penicillin biosynthesis to another classical regulating network that is very well investigated in fungi, especially yeasts (e.g.,

Saccharomyces cerevisiae and *Schizosaccharomyces pombe* [Perez & Calonge, 2002], *Candida albicans* [Monge et al., 2006], and *Cryptococcus neoformans* [Gerik et al., 2005]). Members of the family of PKCs are generally serine/threonine kinases that are found exclusively in eukaryotic cells. Many, if not all, PKC enzymes are central components of signal transduction chains (reviewed in Schmitz & Heinisch, 2003). Computer analysis of the *A. nidulans* genome revealed that the fungus contains two putative PKCs. Whereas PkcA looks like a typical fungal PKC, PkcB apparently misses some of the characteristics, making it likely that PkcB only represents a PKC-related protein kinase (Herrmann et al., 2006).

In *S. cerevisiae*, one of the main functions of PKCs is to maintain cell wall integrity (CWI) during growth and morphogenesis and in the face of external challenges that cause cell wall stress (reviewed in Levin, 2005). Essential elements of this CWI pathway include a set of dedicated plasma membrane sensors, which act through a Rho-type GTPase module upon a range of effector proteins – one of them the PKC orthologue Pkc1p, which affects transcription of a number of cell wall- and morphogenesis-related genes *via* a MAP kinase cascade. It was recently proposed that in *A. nidulans* the PkcA has a function similar to Pkc1p, since a G564R mutation, affecting the regulatory domain of the protein, resulted in a cell-wall defective phenotype. Moreover, a PkcA-GFP protein fusion was localised to sites of active cell wall synthesis (Teepe et al., 2007). This confirmed the results obtained by Herrmann et al. (2006) who observed a growth and sporulation defect under *pkcA* repressing conditions generated by induction of the antisense construct. With the characterisation of the MAP kinase MpkA of *Aspergillus fumigatus*, the existence of the CWI pathway including the respective PkcA orthologue in this filamentous fungus was suggested (Valiante et al., 2008).

However, in filamentous fungi, it is very likely that PKCs have pleiotropic effects and also affect other essential cellular functions. A very recent study showed that the mutation of the *A. nidulans pkcA* gene described by Teepe et al. (2007) conferred resistance to farnesol, a nonsterol isoprenoid that induces apoptosis (Savoldi et al., 2008). The *Neurospora crassa* PKC (NPKC) was shown to also modulate light responses by regulating the blue light photoreceptor white-collar 1 (WC-1) (Franchi et al., 2005). Moreover, since this modulation occurred *via* direct phosphorylation of WC-1, a classical MAP kinase cascade is not necessarily the target of a PKC. Thus, direct phosphorylation of AnBH1 *via* the conserved -**R**QM**S**$^{\mathbf{71}}$E**R**- motif was as conceivable as an indirect influence of PKC. However, microscopic analysis of mutated AnBH1-eGFP fusion proteins (Figs. 16 and 17) indicated that phosphorylation of Ser^{71} at most supports nuclear transport of AnBH1 but is not absolutely necessary; however, a piggy-back transport of AnBH1 into the nucleus in

association with *another* dimerisation partner cannot be ruled out. Therefore, it is more likely that PkcA activates another kinase (cascade) that afterwards phosphorylates AnBH1 using a different amino acid residue, since more putative phosphorylation sites are predicted for the protein (Fig. 13). It will be a future task to analyse these sites, taking into consideration that it may not be the modification of a single amino acid by which the nuclear localisation is regulated (e.g., Komeili & O'Shea, 1999; Faiola et al., 2007). Moreover, this work showed that Ser^{71} is most likely not involved in homo-dimerisation and binding to the *aatA*-promoter *in vitro* (Fig. 18) – a phenomenon that was previously described for other bHLH proteins (Berberich & Cole, 1992; Boyle et al., 1991). This was expected since Ser^{71} is not part of the bHLH motif of AnBH1 (Caruso et al., 2002) that is responsible for dimerisation and DNA binding (Massari & Murre, 2000). Finally, although the re-annotated version of the protein, designated AnBH1f, showed the same properties in terms of calphostin C dependent nuclear localisation and regulation of *aatA* gene expression (Wolke, 2007), it is conceivable that the presence of the N-terminal zinc finger domain is not accidental but might play a role in so far unknown regulatory pathways involving genes and proteins unrelated to penicillin biosynthesis. Since deletion of the *anbH1* gene is apparently lethal (Caruso et al., 2002), a more essential role of the encoded protein has to be assumed.

In summary, PkcA is involved in the penicillin biosynthesis *via* regulation of the nuclear localisation of the transcription factor AnBH1. Furthermore, PkcA appears to be involved in other cellular processes (Herrmann et al., 2006; Teepe et al., 2007). Although the exact function of PKCs in filamentous fungi has not been resolved yet, it would be interesting to speculate that if PkcA was also required for maintaining cellular integrity, this would have a direct effect on the penicillin biosynthesis. Then, hydrolysis of the cell wall of *A. nidulans* by competing bacteria could increase the production of penicillin *via* activation of PkcA.

1.3. A speculative theory

With the identification of VeA and PkcA as new players in the regulation of penicillin biosynthesis so far unknown and partially unexpected external signals were added to the network. Light has been known for a long time to regulate VeA, and PkcA most likely is activated under cell wall stress. However, if VeA was actually also involved in maintaining cell wall integrity (CWI) as suggested by the FvVE1 data from *Fusarium verticillioides*, one could speculate about both factors PkcA and VeA acting in concert (i) to ensure CWI and (ii) if CWI is affected by, e.g., attacking bacteria, to increase penicillin production as a self-defence mechanism. Moreover, if light signalling in *A. nidulans* also involved PkcA as it does

in *Neurospora crassa* (WC-1 and NPKC), then there would exist connections between both the light- and the CWI-induced transduction cascade. However, since this is based on limited data obtained from other organisms, it remains a very speculative but tempting scenario.

2. Evolution of penicillin biosynthesis: IAT and AatB

For several reasons, the *aatA* gene holds a special position within the penicillin biosynthesis gene cluster. In contrast to *acvA* and *ipnA*, it has all features of a eukaryotic gene. Furthermore, it encodes the pathway-specific IAT enzyme. However, neither has the *A. nidulans* IAT itself been characterised in detail and compared to the well studied *P. chrysogenum* enzyme, nor was the evolutionary origin of *aatA* investigated by now – although these are important steps on the way to a full elucidation of the penicillin biosynthesis pathway.

2.1. *A. nidulans* IAT and peroxisomes

For *P. chrysogenum* the importance of IAT being localised within functional peroxisomes has been previously shown (Müller et al., 1992). In *A. nidulans*, under conditions that induce penicillin biosynthesis, proliferation of peroxisomes occurs, but at a low rate (Valenciano et al., 1998). Moreover, initial studies of oleate-nonutilising mutants of *A. nidulans* that possess only few peroxisomes indicated less importance for these single membrane organelles in penicillin production (De Lucas et al., 1997). The present study analysed for the first time in detail the contribution of peroxisomes to penicillin biosynthesis in *A. nidulans* and moreover, the key features of the *A. nidulans* IAT.

First, it was important to prove that the *A. nidulans* IAT is located within peroxisomes, and that this localisation is dependent on the atypical PTS1 sequence -ANI (Fig. 21) that was formerly assumed to function as localisation signal (e.g., Lutz et al., 2005). Here, it was shown that this PTS1 sequence is necessary and sufficient to direct an eGFP-IAT fusion to the peroxisomes. By analysing this protein fusion in different *A. nidulans* peroxin mutants, it was furthermore confirmed that the PTS1 – thus, the PexE-dependent import machinery – and not PTS2 transport is responsible for proper localisation of IAT (Fig. 22). The latter would have been rather surprising since the IAT possesses no obvious PTS2 sequence. Although probably similar to the process in *A. nidulans*, due to the lack of equivalent peroxin mutant strains (Kiel et al., 2004) the IAT of *P. chrysogenum* was not analysed in this respect. It is however striking that both organisms possess different PTS1 sequences for their IATs (Fig. 20).

Fig. 45 shows the DNA sequences of *aatA* and *penDE* that encode these targeting sequences in *A. nidulans* and *P. chrysogenum*, respectively. The differences result from a few base pair modifications whose extent is similar to the overall sequence difference of the genes (70.4 % *vs*. 72.9 % identity; Tobin et al., 1990).

Fig. 45. DNA sequence alignment of the 3' ends of the *A. nidulans aatA* gene and the *P. chrysogenum penDE* gene. Sequences encode the PTS1 (framed) and five upstream amino acids of the respective IATs. Amino acids are indicated. Missense alterations are marked in red, silent alterations in yellow, respectively.

It is difficult to speculate about the evolution, i.e., which PTS1 sequence is more similar to the ancestral one. However, it was previously shown that the efficiency of the peroxisomal transport is dependent on the tripeptide that forms the PTS1 (Swinkels et al., 1992) – and, in contrast to *A. nidulans*, the PTS1 sequence of the *P. chrysogenum* IAT (-ARL) belongs to the most efficient ones. It is thus conceivable, that co-evolution of the PTS1 structure along with the necessity of a peroxisomal localisation of IAT for penicillin production led to the interdependence observed for *P. chrysogenum* (Müller et al., 1992). Consequently, for *A. nidulans* a less efficient PTS1 sequence may have caused the necessity of an enzyme that is also functional in the cytoplasm (see below) and *vice versa*. In addition, this reduced restriction to a specific cellular compartment might be one reason for its lower overall penicillin yield in comparison to *P. chrysogenum*.

In *A. nidulans*, whenever localisation of IAT was affected due to mutation of peroxins or of the enzyme's PTS1 sequence, the penicillin titre was decreased but mutants were still able to produce at least about 50 % of the wild-type penicillin level (Table 6; Figs. 23 and 24). This was also true for mutants that did not possess functional peroxisomes (*pexC::bar*, *pexF23*). *A. nidulans* strains carrying a mutated IAT (AatA$^{\Delta PTS1}$) or had only low numbers of peroxisomes (*pexK*Δ) produced about 75 % of the wild type level. Thus, peroxisomal localisation of IAT – and also of other peroxisomal proteins involved in penicillin biosynthesis, as shown by the PTS receptor mutants *pexE*Δ (PTS1) and *pexG14* (PTS2), respectively – is beneficial but not absolutely required for penicillin production *per se* in this fungus. Because the corresponding peroxin mutants have not been isolated, this has not been analysed that detailed for *P. chrysogenum*, but the data from the mislocalised IAT leading to zero penicillin production (Müller et al., 1992) has already indicated a very different situation.

By contrast, the mislocalised IAT of *A. nidulans* reduces but does not eliminate penicillin biosynthesis (Fig. 24). With AatB a cytoplasmic IAT-like protein involved in penicillin production was identified (see results chapter 4). However, it could be ruled out by the analysis of the *A. nidulans* double mutant $A^{\Delta PTS1}$/B-disr that residual penicillin production of a strain with a mislocalised IAT was only due to AatB activity (Fig. 33). Therefore, and in sharp contrast to *P. chrysogenum*, in *A. nidulans* a mislocalised IAT, despite reduced activity, is still functional. This discrepancy might result from the differences within the amino acid sequence of both proteins (Fig. 20) that could affect enzyme activities, e.g., by modifying the active centre yielding a more tolerant IAT in *A. nidulans*. However, several studies have shown that known amino acid residues that are important for the different activities of IAT are conserved in both enzymes, e.g., the thioesterase domain in the vicinity of Ser^{309} (Alvarez et al., 1993) that was also suggested to be involved in substrate acylation (Tobin et al., 1994), Ser^{227} associated with cleavage and acyltransferase activity (Tobin et al., 1994) likely due to the involvement in binding of isopenicillin N (IPN) (Fernández et al., 2003), and Thr^{105} also associated with both cleavage and activity (Tobin et al., 1993). Despite these similarities, the two IATs still differ in 23.5 % of their amino acid sequence (Tobin et al., 1990), which may explain different tolerances with respect to the cellular environment.

Moreover, whereas in *A. nidulans* peroxisomal mutants are viable (Hynes et al., 2008), the deletion of the respective peroxins in *P. chrysogenum* is most likely lethal (Kiel et al., 2000; Kiel et al., 2004). Thus, alterations of the peroxisomal metabolism may be more stringent regulated in *P. chrysogenum*. Concerning the PexK peroxin that is involved in peroxisome proliferation, data for both organisms are available. They again suggested the major importance of the organelles for penicillin production in *P. chrysogenum* (twofold increase when the orthologue Pc-Pex11p is overproduced; Kiel et al., 2005) – and the comparatively lower importance for *A. nidulans* (reduction to 75 % of wild-type levels in a deletion strain; Fig. 23), although overexpression of a gene cannot be directly compared with deletion of a gene.

Besides IAT, in *A. nidulans* one of the proteins that is also located in peroxisomes could be AN7631.3, the putative homologue of the *P. chrysogenum* phenylacetyl-CoA ligase Phl, possessing a putative PTS1 sequence (-SKL). In *P. chrysogenum*, this enzyme was shown to be involved in penicillin biosynthesis most likely by supplying IAT with the substrate phenylacetyl-CoA (Lamas-Maceiras et al., 2006; indicated by PCL in Fig. 19). Wang et al. (2007) identified a second Phl (PhlB), also containing a PTS1 sequence and being able to catalyse the same reaction. However, for *A. nidulans* a *blastp* analysis using the

P. chrysogenum Phl protein sequence as query yielded 16 hits in total (Fig. 46). In contrast to AN7631.3 having the highest score, two other proteins, AN2549.3 (-AMN) and AN3490.3 (-RNR), do not possess a conserved PTS1 sequence. Although this does not reveal anything about their subcellular localisation, it makes the presence of cytoplasmic Phl-like enzymes likely that could provide a mislocalised IAT with acyl-CoA precursors. Thus, the IPN synthesised by the cytoplasmic IPNS and a mislocalised but functional IAT would enable penicillin biosynthesis in the cytoplasm in *A. nidulans*. If such putatively cytoplasmic Phl-like enzymes do not exist in *P. chrysogenum*, this would explain the lack of penicillin production when IAT was mislocalised in this fungus.

Blast Output

Phl

Summary of Hits by Genome				Show hits from all assemblies		
A. nidulans				16		
Target	**Score (Bits)**	**Expect**	**Alignment Length**	**Identities**	**Positives**	
A. nidulans: AN7631.3: conserved hypothetical protein	742.651	0.0	581	379	458	- SKL
A. nidulans: AN2549.3: conserved hypothetical protein	413.305	0.0	566	218	333	- AMN
A. nidulans: AN3490.3: conserved hypothetical protein	410.994	0.0	582	230	346	- RNR
A. nidulans: AN5990.3: conserved hypothetical protein	323.168	0.0	553	212	315	- AKL
A. nidulans: AN11034.3: conserved hypothetical protein	305.449	0.0	568	203	305	- ARL

Fig. 46. Top five hits of the *A. nidulans blastp* analysis using the *P. chrysogenum* Phl amino acid sequence (Lamas-Maceiras et al., 2006) as query. The blast search yielded 16 hits (indicated). The table shows the blast output of the *Aspergillus* database of the BROAD Institute used for this analysis. On the right-hand site, the C-terminal tripeptide of each protein hit is indicated. Not all tripeptides describe conserved PTS1 sequences.

It is obvious that the contribution of peroxisomes to penicillin biosynthesis in *A. nidulans* is more than providing a better environment for IAT function, since absence of peroxisome formation or of the essential PTS1 transport led to a more severe reduction of the penicillin titre than mislocalisation of the IAT alone. Therefore, compartmentalisation of the final step seems to be advantageous with respect to the yield of the whole process. It is conceivable that keeping enzymes and substrates in closer proximity or facilitating the catalysed reactions, e.g., by the slightly alkaline pH (van der Lende et al., 2002) or a more hydrophobic milieu, could have increased efficiency of hydrophobic penicillin production. However, this work showed that the peroxisomal localisation of the IAT in *A. nidulans* is just beneficial whereas in *P. chrysogenum* it is essential for penicillin production. Despite the lack of equivalent data from *P. chrysogenum*, it can be assumed that this is also true for the presence of functional peroxisomes which are not absolutely required in *A. nidulans*.

The up to 30-fold higher level of penicillin production by *P. chrysogenum* wild-type strains compared to *A. nidulans* wild-type strains is often explained by the inability of the *A. nidulans* IAT to undergo self-processing of the 40 kDa preprotein to yield the 11 kDa and 29 kDa subunits of the active heterodimer (e.g., in Garcia-Estrada et al., 2008). This was proposed by Fernández et al. (2003) who did not observe processing when the *A. nidulans* IAT was produced in different heterologous hosts. Moreover, Western blot analyses of *P. chrysogenum* and *A. nidulans* crude extracts using antibodies against the respective IATs showed that the *P. chrysogenum* IAT was already fully processed after 48 h of incubation under penicillin producing conditions, whereas the *A. nidulans* enzyme remained in the 40 kDa form for at least 96 h of incubation. Although processing was shown to be a prerequisite for an active enzyme in *P. chrysogenum* (Tobin et al., 1993; Tobin et al., 1994), Fernández et al. (2003) suggested the *A. nidulans* 40 kDa IAT to be the active enzyme, since they only observed enzymatic activity with the full-length protein produced in the heterologous *E. coli* host, but never with mixtures of the subunits. However, the unexpected finding of the present study at least should raise some concerns about this proposed processing inability of the *A. nidulans* IAT. In Western blot analysis of crude extracts from an *A. nidulans* strain that produced an eGFP-IAT fusion protein, the signal corresponding to the unprocessed eGFP-IAT was found to be only very faint, whereas the one corresponding to the cleaved protein was clearly present (Fig. 34). Hence, these data were highly indicative of an *in vivo* processing of the *A. nidulans* IAT under the applied conditions, although further experiments are necessary to evaluate these results.

2.2. *aatB* and the evolution of *aatA*

Where does the pathway-specific *aatA* gene come from? Data obtained by this work to answer this interesting question may be also very helpful in terms of more general aspects of the evolution of secondary metabolism genes. The biosynthesis of the β-lactam antibiotics penicillin and cephalosporin is an advanced model to investigate evolution because these compounds are synthesised by both eukaryotes (filamentous fungi) and prokaryotes (bacteria) (reviewed in Brakhage et al., 2004). In particular, the organisation of the biosynthesis genes in clusters in both kingdoms and the notion that the fungal penicillin biosynthesis genes *acvA* and *ipnA* lack introns argued for horizontal gene transfer (HGT) from an ancestral prokaryote to a common ancestor of the β-lactam synthesising fungi. For the third gene of the penicillin biosynthesis gene cluster, *aatA*, however, no bacterial ancestor gene had been identified.

Having all features of a eukaryotic gene led to the assumption that the *aatA* gene was recruited during evolution to the *acvA-ipnA* cluster (summarised in Brakhage et al., 2005).

With *aatB* a novel gene involved in the penicillin biosynthesis was identified in this work– a finding that may help elucidating the evolution of the gene cluster. The role of *aatB* in penicillin biosynthesis was supported by the surprising findings that the *A. nidulans aatA*-disruption strain still produced small amounts of penicillin V (Fig. 26). Similar analyses of a mutated locus of the *penDE* homologue in *P. chrysogenum* revealed no significant levels of penicillin G (Cantoral et al., 1993), again substantiating certain differences between both penicillin producers. Since IAT possesses the key enzymatic activities necessary to convert IPN *via* acyl-CoA : 6-aminopenicillanic acid (6-APA) to a hydrophobic penicillin (Alvarez et al., 1993), it was assumed that this enzyme was necessary and sufficient for catalysing this reaction. However, this work showed that in *A. nidulans* a second gene, *aatB*, is necessary to reach wild-type levels of penicillin production and, moreover, only a disruption of both the *aatA* and the *aatB* gene could reduce penicillin titres below detection level (Fig. 31B). This was supported by the fact that the *aatB* gene (locus AN6775.3) was the only hit for a *blastn* search with the *aatA* sequence as query (Fig. 27A). Thus, a novel gene (*aatB*) capable of partially replacing the *aatA* gene was identified.

Both genes *aatA* and *aatB*, and also the encoded proteins IAT and AatB, respectively, have various features in common, i.e., similar sizes, similar exon distributions and domain structures (except for the PTS1 sequence; Fig. 27B+C), and a similar processing capability (Fig. 34). Both genes also show expression patterns similar to each other (although with a different intensity) and to genes of primary metabolism (Fig. 36) – in this respect they are clearly distinguishable from secondary metabolism genes (Brakhage et al., 1992; Litzka et al., 1995). Moreover, their promoters possess binding sites for the same transcription factors, AnCF and AnBH1 (Fig. 37), which not only bind to these promoter sequences *in vitro* (Figs. 38 and 39), but also have a regulatory relevance *in vivo* (Fig. 40). However, along with the unequal contribution of IAT and AatB to penicillin biosynthesis (compare Fig. 26 with 31A), the major difference was observed for the subcellular localisation of the proteins due to the PTS1 sequence that is not present in AatB, thus localising the enzyme to the cytoplasm (Fig. 32). These data indicate that *aatA* and *aatB* are paralogues derived from duplication of a common ancestor gene with one counterpart being recruited to the *acvA-ipnA* cluster and becoming the *aatA* gene that is present today. In general, gene duplication is considered as an ordinary event in evolution. It is the driving force for creating new genes in genomes: at least 50% of prokaryotic and over 90% of eukaryotic genes result from gene duplication events

followed by specialisation (Teichmann & Babu, 2004). This is presumably also true for the evolution of *aatA*, and thus of the penicillin biosynthesis gene cluster. Even stronger indicators of *aatA* and *aatB* being paralogues are the high similarity at both the nucleotide and amino-acid level, along with the mentioned almost identical exon distribution.

Data obtained by phylogenetic analysis showed that *aatB*-like genes are consistently distributed within the fungal kingdom (subphylum Euascomycota), whereas *aatA*-like genes are only found as part of the penicillin biosynthesis cluster forming an own clade within the tree with only four members (Fig. 28). The question of when and how often the duplication event occurred cannot be satisfactorily answered at the moment. If it happened before the splitting of the genera *Aspergillus* and *Penicillium*, why is the *aatA* gene missing in most of the Aspergilli, and why, despite the then obvious evolutionary distance, do the *aatA* homologues show such a high identity of about 80% of the amino acid sequence? And if it had occurred later, how was it transferred to the particular species if not by HGT with the other penicillin biosynthesis genes, as recently reported for another secondary metabolism gene cluster (Khaldi et al., 2008)? Because of the high sequence identity of the *aatA*-encoded proteins, the consistent association of the *aatA* gene with the *acvA-ipnA* gene cluster and the fact that a duplicated *aatB* gene can also be found in some penicillin non-producing fungal species (i.e., *Aspergillus terreus*; Fig. 28), an independent duplication event in all four species possessing an *aatA* homologue is rather unlikely. It is conclusive that HGT of the whole gene cluster between fungal species followed the proposed interkingdom HGT of *acvA-ipnA* from bacteria to fungi and the recruitment of a common ancestor of *aatA* and *aatB* (depicted in the model in Fig. 47). However, given the low number of species encoding the cluster, this second, *intra*kingdom HGT cannot be proven unequivocally.

It has been shown for *S. cerevisiae* that in the case of gene duplications the regulatory elements tend to be co-duplicated with the genes, although common transcription factor binding sites may be lost as a function of evolutionary time (van Noort et al., 2004; Hughes & Friedman, 2007). For *aatA* and *aatB* the presence of common regulatory *cis*-elements in their promoters was indicative of shared *trans*-acting factors (Fig. 37). Accordingly, the present study showed by EMSA and SPR analyses that both *aatA* and *aatB* promoter were bound by the same regulators AnCF and AnBH1f *in vitro* (Figs. 38 and 39). Interestingly, the regulator proteins showed similar relative binding kinetics independent of the promoter sequence used as DNA probe, i.e., binding of AnCF occurred with an approximately 13 times higher affinity than binding of AnBH1f (K_D values of 1.82 nM *vs.* 22.7 nM for the *aatA* promoter and 5.48 nM *vs.* 73 nM for the *aatB* promoter, respectively). The observed K_D values for AnCF

were in the range of those previously found for this regulator (Hortschansky et al., 2007). Concerning AnBH1f, this was the first study that investigated binding of a bHLH protein by SPR analysis, but compared to other regulators, a lower binding-affinity might be a common feature of these transcription factors. It remains a matter of speculation whether the differences observed *in vitro* for the absolute K_D values and *in silico* for number and arrangement of the respective binding sites can account for the differences observed *in vivo* for expression of both genes, *aatA* and *aatB*. It was shown for the *aatA* promoter (Caruso et al., 2002), that the overlapping binding sites led to mutual exclusive binding of AnCF and AnBH1 – which was confirmed in this study (Fig. 39A) – and thus to oppositional effects on *aatA* expression. Furthermore, the generally higher mRNA-steady-state level of *aatA* under fermentation conditions (Fig. 36) might be one reason for its higher impact on penicillin biosynthesis compared to *aatB*, since in AMM both genes show similar mRNA-steady-state levels. The finding, that both promoters are not only bound by AnCF and AnBH1f *in vitro*, but that these regulators also influence *aatA* and *aatB* gene expression in a different way *in vivo* (Fig. 40), requires further investigations.

The conclusions concerning shared transcriptional regulators extended the hypothesis of the duplication event yielding *aatB* and *aatA*: not only was the ancestral structural gene duplicated, but also the respective regulatory *cis*-acting sites. The fact that those sites are present in the respective putative homologues in other organisms (Table 7) also supported this theory. The recruitment of one duplicate to the penicillin biosynthesis gene cluster becoming *aatA* was thus accompanied by the recruitment of the respective *trans*-acting factors AnCF and AnBH1f (see model in Fig. 47), although one can only speculate about how these regulators were recruited to the other genes of the cluster, i.e., AnCF to *ipnA*. It is interesting to note, however, that fungal secondary metabolism gene clusters do not need to contain a regulatory gene. Similar to the β-lactam biosynthesis gene clusters in eukaryotes (Schmitt et al., 2004; Brakhage et al., 2004), no regulatory gene was found within the gene clusters for biosynthesis of ergot alkaloids (Haarmann et al., 2005), fumonisin (Proctor et al., 2003), or gibberellins (Tudzynski, 2005). In such cases, recruitment of global regulators is a prerequisite for the functional establishment of gene clusters. It has already been demonstrated that the penicillin biosynthesis gene cluster by itself is able to confer penicillin production capability to otherwise non-producing filamentous fungi and that, therefore, non-pathway-specific regulators need to be involved (Smith et al., 1990).

The *aatB* gene and its encoded function appear to be not essential for *A. nidulans* because disruption mutants are viable. It is likely that AatB represents an acyl-CoA

transferase because it has the corresponding AAT domain (Fig. 27C) and, moreover, this is the function of IAT that is required for penicillin biosynthesis. Although the important amino acid residue Ser^{309} (Ser^{308} of the *A. nidulans* AatB), that is associated with activity of the *P. chrysogenum* IAT (Tobin et al., 1994), is conserved in AatB (Fig. 27C), its lower impact on penicillin biosynthesis may result from the S228T substitution corresponding to Ser^{227} of the IAT proteins. This residue is conserved within the IATs including *Aspergillus oryzae* (Fig. 29C), and assumed to be involved in IPN binding (Fernández et al., 2003). Thus, the required nucleophilic (Tobin et al., 1994) threonine residue at this position may not alter acyl-transferase activity in general, but possibly modifies substrate specificity resulting in less IPN conversion rates. This leads to the very speculative assumption of a specialisation / adaptation process of the IAT proteins, but to support this theory more biochemical data are necessary – particularly with regard to the presence of a corresponding serine residue in the putative AatB homologues of *P. chrysogenum* and *A. oryzae* (Fig. 29C).

Furthermore, the presence of AatB also in non-penicillin producing fungi (Fig. 28) suggests an additional, more general role independent of the penicillin production; a function that was probably fulfilled by the ancestral protein. Preliminary investigations to get a clue to this function were performed in this study. Growth tests of *A. nidulans* mutant strains on different fatty acids as sole carbon sources led to the conclusion that AatB is – if at all – only marginally involved in fatty acid metabolism. Surprisingly, absence of the protein conferred a higher resistance to usually toxic concentrations of phenylacetic acid (PA) and benzoic acid (BA), the latter becoming even more evident when also IAT was lacking (Fig. 41). In *A. nidulans*, the toxicity of PA can be avoided by two competing mechanisms, (i) catabolic degradation *via* homogentisate (Mingot et al., 1999) and (ii) detoxification by incorporation into penicillin – proposed as an example of the detoxification hypothesis for the origin of several antibiotic biosynthesis pathways (Dhar & Khan, 1971). Blockage of the catabolic pathway in *P. chrysogenum* is most likely one reason for the higher penicillin yields observed in this fungus (Rodriguez-Saiz et al., 2001). The results obtained by the present study indicate that AatB possesses no detoxifying potential for PA, and for BA neither. On the contrary, it apparently contributes to toxicity by a so far unknown mechanism. However, the connection to the mode of action of such weak acids, i.e., impairing transport systems by dissipation of the transmembrane pH gradient (Hunter & Segel, 1973), is not obvious at first glance. Therefore, further experiments are indispensable to elucidate this role of AatB.

After the gene duplication event, two proteins with the same enzymatic capabilities were present in the organism – one became AatB, the other IAT. It is conceivable that the IAT

protein evolved faster to adapt to its "new" function because of the occurrence of ACVS and IPNS activity and the presence of the substrate IPN in the cells. Therefore, during evolution, the *aatA* gene and the encoded IAT must have undergone specialisation, because overexpression of the *aatB* gene only partially complemented an *aatA* disruption with respect to penicillin production (Fig. 35). Such specialisation events can happen either at the level of regulation (Louis, 2007), or at the level of protein structure with the gain of more functional domains leading to a beneficial function termed neofunctionalisation (Lynch & Force, 2000).

The most obvious feature that discriminates IAT from AatB is the PTS1 sequence leading to peroxisomal localisation of IAT, whereas AatB is located in the cytoplasm. Interestingly, this PTS1 sequence was sufficient to localise AatB to peroxisomes (Fig. 32C). Previously, for derivatives of the *P. chrysogenum* production strain Wis54-1255 it was shown that strains with a mislocalised IAT were unable to produce penicillin, although the IAT enzyme was active in *vitro* (Müller et al., 1992), indicating an important role of peroxisomal localisation for IAT in this fungus (see also chapter 2.1. of the discussion). As shown by the present study (Fig. 35), in an *A. nidulans aatA*-disruption strain that overproduced the cytoplasmic AatB the penicillin production was significantly increased compared to a scenario without *aatB* overexpression. The penicillin titre was increased even more by adding the PTS1 sequence of the IAT to AatB, resulting in peroxisomal localisation of the modified AatB protein. Therefore, and as already shown by this work for the IAT (Fig. 24), in *A. nidulans* the peroxisomal localisation of the catalysing enzyme is advantageous to increase penicillin production but not essential for production *per se*. Because production of the hydrophilic cephalosporins apparently does not require peroxisomal localisation of the involved enzymes either (Evers et al., 2004), it is probably only advantageous for the production of the hydrophobic penicillins to have the last step catalysed by the IAT within the peroxisomes. Therefore, it is very likely that the acquisition of a PTS1 sequence along with the duplication and recruitment events was an important step in evolution of the penicillin biosynthesis gene cluster (see model in Fig. 47).

The question remains, why in a eukaryote the ancestor of the *aatA* gene was recruited to the *acvA-ipnA* cluster. It is unlikely that the simultaneous regulation by *trans*-acting factors enforced the clustering, since in eukaryotes regulators can control the expression of dispersed genes as effectively as clustered genes (Keller & Hohn, 1997). It was previously suggested that clustering has only a selective advantage for the cluster itself (Walton, 2000), especially if selection pressure is relatively weak as for genes encoding proteins with nonessential functions, e.g., in secondary metabolism, because, if clustered, genes could be distributed by

HGT more easily. Thus, physical proximity was no selective benefit for the donor organism, but provided a reasonable chance of conferring a new selective advantage to a "naïve" genome (Lawrence & Roth, 1996), which would promote the survival of both the new host and the transferred genes. Therefore, the gene cluster could be considered as selfish. According to this theory, one can assume that in a common ancestor the duplicated *aatB*-like *aatA* gene was recruited to the *acvA-ipnA* gene cluster to allow the transfer of the whole cluster to non-producing organisms by HGT. As mentioned above, the recruitment could not have occurred before, but rather after the splitting of the genera *Penicillium* and *Aspergillus* and also after *Aspergillus* species had evolved. However, this remains only one of possible scenarios, since the moment of the gene duplication is hardly predictable. On the other hand, a duplication event before the splitting of the genera would arise the question why the *aatA* gene, and thus the whole cluster, was lost in the other species.

In addition to the hypothesis that maintaining clustering in eukaryotes may be a prerequisite for promoting HGT, more recent studies have indicated that clustering, and thus locating the genes of a secondary metabolism pathway in close proximity, enables chromatin-based mechanisms of transcriptional control. The (histone-) methyl transferase LaeA (Bok & Keller, 2004; see also Fig. 44) and the histone deacetylase HdaA (Shwab et al., 2007), two global regulators of chromatin modification in *A. nidulans*, were shown to affect expression of the clustered sterigmatocystin and penicillin biosynthesis genes as well as other secondary metabolism gene clusters. Therefore, the advantages of common, chromatin-based regulation could be a good reason for *aatA* being recruited to *acvA* and *ipnA* to form the gene cluster that is present today, although this hypothesis still gives no explanation of a possible mechanism of the cluster formation.

2.3. A model of evolution of the penicillin biosynthesis gene cluster

It is the predominant hypothesis, that the first two genes of the penicillin biosynthesis gene cluster, *acvA* and *ipnA*, were obtained by interkingdom HGT from bacteria, e.g., a *Streptomyces* species (see also Fig. 3 of the introduction), to an ancestor of the β-lactam producing fungi, and thus of *A. nidulans* (HGT I). This work showed that the novel gene *aatB* is involved in penicillin biosynthesis in *A. nidulans* and that this gene is linked, most likely by a duplication event, to the ancestor of the third penicillin biosynthesis gene *aatA*. Furthermore, the data suggest that in the course of assembly of *aatA* and *acvA-ipnA* into a single gene cluster – which is an established theory since there are no bacterial homologues of the obviously eukaryotic *aatA* gene – a simultaneous recruitment of the transcriptional

regulators occurred. Therefore, this work extends the model of evolution of the penicillin biosynthesis gene cluster by recruitment of a biosynthesis gene and its *cis*-acting sites upon gene duplication. Moreover, the phylogenetic analyses indicate that the often proposed intrakingdom HGT of the whole cluster from a penicillin-producing fungus to non-producers (HGT II) is very likely but cannot be proven unequivocally, and thus, requires further investigation. Fig. 47 includes the new data obtained in this work into the prevalent model of evolution of the penicillin biosynthesis gene cluster.

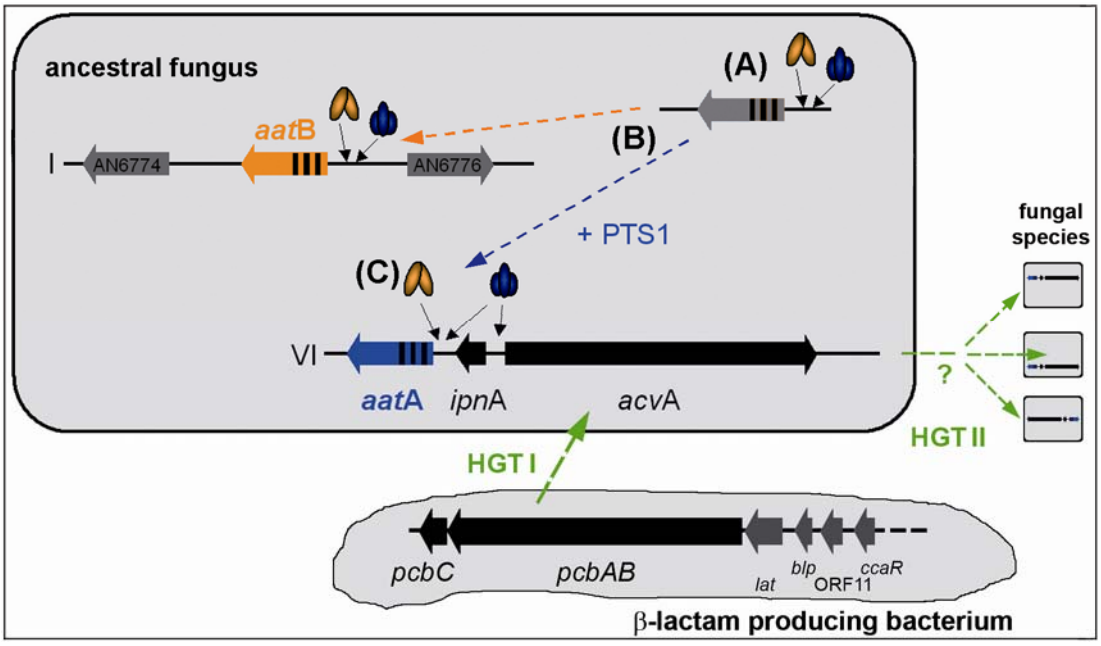

Fig. 47. An extended model of the evolution of the penicillin biosynthesis gene cluster in a putative ancestor of *A. nidulans*, with focus on the data obtained in the present study. Unrelated uncharacterised genes are named according to their locus ID. Interkingdom (I) and intrakingdom (II) HGTs are indicated (see text for details). Dashed arrows indicate evolutionary processes. **(A)** An ancestral gene of both *aatA* and *aatB* was regulated by AnCF (pictured in blue) and AnBH1f (orange). **(B)** The ancestral gene was duplicated. One copy became after modification *aatB*, the other one *aatA*. The *aatA* gene was associated with the *acvA*-*ipnA* gene cluster that was most likely obtained by HGT I, and underwent further modification processes, e.g., acquirement of a PTS1 sequence. **(C)** It is conceivable that AnCF and AnBH1f were brought to the gene cluster *via* the *cis*-acting DNA elements of the *aatA* promoter and, in the case of AnCF, became also involved in the regulation of *ipnA* gene expression. HGT II would explain the occurrence of the gene cluster in only particular fungal species.

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LIST OF PUBLICATIONS

Parts of this thesis are included in the following publications:

Spröte P, Hynes MJ, and Brakhage AA (2008): Contribution of peroxisomes to penicillin biosynthesis of *Aspergillus nidulans*. Submitted.

Spröte P, Hynes MJ, Hortschansky P, Shelest E, Scharf DH, Wolke SM, and Brakhage AA (2008): Identification of the novel penicillin biosynthesis gene *aatB* of *Aspergillus nidulans* and its putative evolutionary relationship to this fungal secondary metabolite gene cluster. *Molecular Microbiology* 70:445-461.

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Brakhage AA, Spröte P, Al-Abdallah Q, Gehrke A, Plattner H, and Tüncher A (2004): Regulation of penicillin biosynthesis in filamentous fungi. *Advances in Biochemical Engineering / Biotechnology* 88:45-90.

Parts of this thesis were presented at the following scientific conferences:

Spröte P, Hynes MJ, Hortschansky P, and Brakhage AA (2008): Evolution of fungal secondary metabolism genes: *aatB* of *Aspergillus nidulans* – the putative ancestor of *aatA*. *Poster presentation at the "9th European Conference on Fungal Genetics" (Edinburgh, UK).*

Spröte P, Hynes MJ, and Brakhage AA (2007): Identification of a novel penicillin biosynthesis gene. *Talk at the VAAM Annual Conference (Osnabrück, Germany).*

Spröte P, Hynes MJ, and Brakhage AA (2007): Identification of AatB: new insights into the evolution of the penicillin biosynthesis pathway of *Aspergillus nidulans. Poster presentation at the "24th Fungal Genetics Conference" (Monterey, CA; USA).*

Herrmann M, Spröte P, and Brakhage AA (2005): Protein kinase C2 of *Aspergillus nidulans* is involved in the penicillin production. *Poster presentation at the 7th VAAM Symposium "Molecular Biology of Fungi" (Bochum, Germany).*

Spröte P, and Brakhage AA (2005): The developmental regulator velvet A represses the penicillin biosynthesis of *Aspergillus nidulans*. *Talk at the 7th VAAM Symposium "Molecular Biology of Fungi" (Bochum, Germany).*

SUPPORT

The experimental work for this thesis was carried out at the Department of Molecular and Applied Microbiology of the Leibniz Institute for Natural Product Research and Infection Biology (HKI Jena), and at the Department of Genetics of the University of Melbourne.

This work was financially supported by *Deutsche Forschungsgemeinschaft* (SPP 1152 "Evolution of Metabolic Diversity") and a short-term scholarship for PhD students granted by *Deutscher Akademischer Austauschdienst* (DAAD).

DANKSAGUNG – ACKNOWLEDGEMENTS

Mein erster Dank gilt meinem Doktorvater Prof. Dr. Axel A. Brakhage. Für ihn war und ist die Penicillinbiosynthese eine Herzensangelegenheit – und damit hat er mich schnell angesteckt. Seine hilfreichen Anregungen, aber auch die konstruktive Kritik haben mich stets vorangebracht. Zugleich waren die experimentellen Freiheiten und das mir entgegengebrachte Vertrauen eine wertvolle Unterstützung. Ganz besonders möchte ich mich dafür bedanken, dass er es mir ermöglicht hat, einen Teil dieser Arbeit in Melbourne durchzuführen.

I am very grateful to Prof. Dr. Michael J. Hynes and the whole HDA lab in Melbourne for the warm welcome and the marvellous time I had "down under" – both inside and outside the lab. They introduced me to the peroxisomal world, shared my excitement about the discovery of *aatB*, and also after my return to Germany I could always count on their support. A very special thank to Kathrin for her help and for sharing many ups and downs.

Mein Dank geht auch an die Mitarbeiter des Instituts für Mikrobiologie der Leibniz-Universität Hannover, in welchem diese Arbeit ihren Anfang fand. Vor allem Dr. Hans Plattner wird mir als langjähriger Mentor immer in Erinnerung bleiben.

Insbesondere bei Dres. Peter Hortschansky, Olaf Kniemeyer, Ekaterina Shelest, Robert Winkler und Matthias Brock möchte ich mich für die Hilfe bei den verschiedensten Analysemethoden bedanken, die in dieser Arbeit zur Anwendung kamen. Dr. Martina Herrmann danke ich für die erfolgreiche Zusammenarbeit bei der Untersuchung der PkcA. Technische Unterstützung erhielt ich vor allem von Christina Täumer, Andrea Perner und Sylke Fricke – auch dafür vielen Dank!

Die angenehme Atmosphäre im Labor (danke Sebastian!), im Büro und in der gesamten Abteilung MAM hat mir den Arbeitsalltag stets sehr erleichtert. Dafür danke ich allen Kollegen ganz herzlich – auch denen der jetzigen Nachwuchsgruppe MBP. Ich weiß, dass diese Hilfsbereitschaft und dieser Teamgeist nicht selbstverständlich sind.

Mein besonderer Dank gilt Alexander, Thorsten, Jeannette, Judith und André – sie haben mich jahrelang als Kollegen begleitet und sind dabei echte Freunde geworden. Ein großes Dankeschön an Nadine, Sabine und Stefan stellvertretend für die Menschen, die mich daran erinnert haben, dass es auch ein Leben fernab von Penicillin und Schimmel gibt.

Die letzten Zeilen gehören meiner Familie: meinen Schwestern und meinen Eltern. Ohne ihre fortwährende Unterstützung und Motivation, vor allem während der Zeit in Australien, wäre diese Arbeit nicht möglich gewesen. Danke, dass ich in jeder Hinsicht auf Euch zählen konnte!

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EIGENSTÄNDIGKEITSERKLÄRUNG

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt.

Bei der Auswahl und Auswertung des Materials haben mich die in der Danksagung meiner Dissertation genannten Personen unterstützt. Personen, die bei der Anfertigung der Publikationen beteiligt waren, sind in der Publikationsliste angegeben.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Jena, 15. Dezember 2008

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